

The Role of the NOD2 gene in the Pathogenesis of Crohn's Disease

by

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Declaration

I, Teresa Alice Chalmers-Watson, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. I can confirm that I performed all experiments, with assistance from Dr Annabel Bromfield, Dr Sanjay Lala and Dr Satish Keshav.

ABSTRACT

Introduction: Crohn's disease is characterised by an abnormal inflammatory response possibly induced by components of enteric bacteria, in genetically susceptible individuals. Mutations in the *NOD2* gene are strongly associated with Crohn's disease, although the mechanisms by which these mutations cause Crohn's disease remain unknown. Peripheral blood monocytes (PBMC), a key component of the innate immune system, highly express *NOD2*, as do intestinal epithelial Paneth cells. The ligand for *NOD2* is Muramyl dipeptide (MDP), a component of bacterial peptidoglycan. MDP has been shown to be a powerful priming agent for subsequent stimulation by lipopolysaccharide (LPS) in cell lines and this effect has been linked to *NOD2*. Studies of primary mononuclear cells in Crohn's disease comparing the functional effects of the *NOD2* mutations had not previously been reported.

Aims: To determine the effect of inherited mutations in the *NOD2* gene on the cellular responses of freshly extracted PBMC to MDP and other bacterial ligands including *mycobacteria*. To examine the effect of MDP pre treatment 'priming' on PBMC responses to subsequent LPS stimulation in both normal and Crohn's disease affected patients expressing wild type and mutant *NOD2* proteins.

Methods: PBMC from healthy controls (n=12), and Crohn's disease affected patients who were genotypically either wild type (n=12), heterozygous (n=11) or homozygous (n=5) for the common disease-causing *NOD2* mutations. PBMC were stimulated with bacterial products *in vitro*, with or without prior stimulation or 'priming' with MDP. The transcription of selected cytokine genes was determined by real time quantitative RT-PCR

Results: MDP is a weak stimulant of inflammatory responses in PBMC whereas LPS evoked much stronger responses. Responses to MDP were particularly reduced in PBMC homozygous for the *NOD2* mutations. Priming with MDP reduced the inflammatory response of normal PBMC to subsequent LPS stimulation. In PBMC carrying two mutant *NOD2* alleles this modulatory effect was reversed and MDP priming caused the inflammatory response to be enhanced.

Conclusion: MDP priming significantly modulates responses of monocytes to LPS. This effect is altered in patients with Crohn's disease – possibly related to mutations in the *NOD2* gene. This modulatory effect may explain in part the pro-inflammatory consequence of mutations in the *NOD2* gene and could provide mechanistic understanding of how mutations in the *NOD2* gene may cause Crohn's disease.

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Chapter One

1.0 Introduction

Inflammatory bowel disease (IBD) is a chronic relapsing, idiopathic inflammatory disorder of the gastrointestinal tract. The two main forms of IBD - Crohn's Disease (CD) and ulcerative colitis (UC) have many similarities, but also several distinct clinical and pathological differences. The diagnosis depends on the aggregate constellation of the clinical history, physical findings, endoscopic, radiological, and histological and laboratory findings. These features allow a firm diagnosis to be made in the majority of cases. In 10% of cases where the inflammation is limited to the colon, they are indistinguishable and are categorised as 'indeterminate colitis'

The incidence of Crohn's disease has increased rapidly over the last half of the 20th century in almost all western countries. The aetiology of Crohn's disease remains unknown, although there is an accumulating body of evidence that strongly suggests that CD develops as a consequence of an excessive or unregulated mucosal inflammatory reaction mounted in response to components of intestinal microflora (Bouma 2003). Recently epidemiological and linkage studies suggest that genetic factors play a significant role in determining IBD susceptibility. Most notable is the identification that individuals carrying mutations in the NOD2 gene are predisposed to develop Crohn's disease (Hugot 2001, Ogura 2001). It is postulated that NOD2 proteins, which sense bacterial antigens, play a role in the protective immune responses against pathogens (Inohara 2005). Nonetheless, the reasons why individuals with NOD2 mutations are predisposed to develop Crohn's disease remain unknown.

In the present thesis, I will examine the effect of inherited mutations in the NOD2 gene on cellular responses to bacterial stimuli.

1.1 Crohn's disease

Crohn's disease is an inflammatory disorder affecting any part of the gastrointestinal tract from the oropharynx to the perianal area. It is a chronic disorder that is characterised by alternating periods of active intestinal inflammation and resolution. The incidence of CD has increased exponentially over the last 4 decades whereas no clear trend is identifiable in UC. The most recent measured prevalence of CD was 144 cases per 100,000 in the UK (Rubin 2000). Disease incidence is highest in the developed, urbanised countries. CD is more common in women than men and shows a bimodal age distribution with a major peak at 15-30 years of age, and a lesser one at 60-80 years. (Fellows 1990)

The clinical presentation of Crohn's disease varies and depends on the site, extent and severity of inflammation. The original description of the disease by Crohn, Ginzberg & Oppenheimer in 1932 localised the disease to the ileum (ileitis), however the same inflammatory process may involve any part of the gastrointestinal tract, and it preferentially affects the terminal ileum, followed by the caecum, peri-anal area and colon (colitis). CD is characterised by the presence of segments of normal bowel between affected regions, known as 'skip' lesions. The intersection of linear ulcers with islands of normal mucosa can produce a classical 'cobblestone' appearance. The histology is characteristic with transmural dense infiltration of lymphocytes and macrophages and granulomas are present in 60% of cases. Due to the transmural nature of the inflammation, extra luminal complications such as fistulae, either enterocutaneous or intestinal fistulae that communicate with other intestinal and pelvic organs may occur.

The major clinical features of CD are abdominal pain, diarrhoea, anorexia, weight loss & fatigue. The complications of the disease are often local resulting from intestinal inflammation and involvement of adjacent structures leading to intestinal strictures, bowel obstruction, abscess formation and fistula formation that may communicate with other intestinal and pelvic organs or develop into enterocutaneous fistula (Keshav 2004)

In IBD, fever, malaise and weight loss may occur due to systemic inflammatory responses, and these symptoms are often more severe in patients with Crohn's disease. Extra-intestinal manifestations of Crohn's disease include arthralgia and arthritis, pyoderma gangrenosum

and erythema nodosum, and iritis and uveitis. Some of these are related to the underlying disease activity. There is currently no curative therapy for Crohn's disease and patients usually require long term therapy with immunosuppressive therapy such as Azathioprine, Mercaptopurine or Methotrexate to reduce intestinal inflammation. Newer biological drugs such as anti-tumour necrosis factor (TNF) antibodies which target the general inflammatory cytokine $\text{TNF}\alpha$, are increasingly being used but these too are limited by limited efficacy and toxicity. Surgical treatment is reserved for treating the complications of CD.

1.1.1 Pathogenesis of Crohn's disease

The aetiology of Crohn's disease remains unknown. However many investigators hypothesise that IBD results from an inappropriate and exaggerated mucosal immune response to normal constituents of the intestinal microflora that is, in part, genetically determined (Podolsky 2002, Bouma 2003). In Crohn's disease, mucosal inflammation is mediated by an excessive T helper 1 ($\text{T}_{\text{H}1}$)-cell response that is associated with increased secretion of interferon γ ($\text{IFN}\gamma$), tumour necrosis factor α ($\text{TNF}\alpha$) and interleukin-12 (IL-12) (Bouma 2003). This contrasts with patients with UC whose mucosa is dominated by CD4^+ lymphocytes with a typical type 2 helper T cell ($\text{T}_{\text{H}2}$) phenotype, characterised by the production of transforming growth factor β (TGF- β), interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-13 (IL-13).

The stereotypical $\text{T}_{\text{H}1}$ cytokines activate macrophages which in turn produce interleukin-12 (IL-12), interleukin-18 (IL-18) and macrophage migration inhibition factor and thus further stimulate $\text{T}_{\text{H}1}$ in a self sustaining cycle. As importantly, activated macrophages produce a potent mix of active inflammatory cytokines including $\text{TNF}\alpha$, interleukin-1 (IL-1) and interleukin-6 (IL-6).

1.1.2 Murine Models of IBD

An important advance in the study of IBD has been the discovery and analysis of murine models of intestinal inflammation which resemble IBD. From these studies Bouma (2003) has proposed seven principles that are important in understanding the pathogenesis of human IBD. (Bouma 2003) The first principle states that entirely different genetic abnormalities can lead to the same clinical features of intestinal inflammation. The second principle states that the host genetic background determines the susceptibility to intestinal inflammation, even when a major genetic defect that predisposes to inflammation is present for example some strains of mice with IL-10 deficiency are highly susceptible to colitis whereas others are resistant. (Mahler 2002)

Experimental colitis does not develop when mouse are kept in a germ free environment (Sartor 1997). This leads to the third principle that states that the normal mucosal microflora is required to establish and maintain the inflammatory response presumed to be by providing one or more antigens or co factors that drive the immune response in a genetically susceptible host. Despite an extensive search no specific pathogenic micro organisms have been associated with any of the mouse models so far. The fourth principle states that experimental inflammation is due to either excessive effector T-cell function or deficient regulatory T-cell function. The former possibility is exemplified in mouse models where TNF is over produced as a result of a deletion in the AU-rich regulatory elements (ARE) (TNF^{ARE} mice) (Kontoyiannis 1999). In human Crohn's Disease, excessive T_H1-cell-mediated inflammation is the best characterised pathophysiologic feature of Crohn's disease. It is not clear however whether mucosal inflammation, may also be caused by a defect in regulatory T-cell function because it is not possible to measure regulatory cell responses with confidence in patients with Crohn's disease.

The fifth principle states that despite a wide variety of causes, intestinal inflammation is either mediated by excessive T helper 1 (T_H1)-cell or T helper 2 (T_H2)-cell responses. This dichotomy between T_H1 and T_H2 inflammation is seen in human IBD where the histopathological features of Crohn's disease resemble those of experimental T_H1 cell mediated colitis, whereas those of UC are most similar to experimental T_H2 cell mediated

colitis and the cytokine patterns that are characteristic of these diseases are in accordance with these distinct mechanisms.

The sixth principle states that the epithelium of the intestinal mucosa has an essential role in the physical separation of potentially stimulating microflora and the reactive cells of the mucosal immune system (Podolsky 1999, Hermiston 1995). The effect of the loss of this barrier function was clearly shown in mice who developed severe inflammation in areas of the intestine where the epithelial cell barrier was disrupted. (Hermiston 1995)

Finally, the seventh principle states that mucosal inflammation can also arise due to specific defects in the innate immune system and genetic abnormalities of innate immunity involving the function of antigen-presenting cells, macrophages, or natural killer cells. (Takeda 1999) In human IBD there is emerging evidence to suggest that defects in innate immunity may predispose patients to IBD. Wehkamp and colleagues (2004) have reported that expression of the antimicrobial peptides alpha defensin 5 (HD5) and 6 (HD6) is lower in patients with Crohn's disease as compared to patients with ulcerative colitis. In Crohn's disease, HD5 and HD6 expression is especially low in those patients carrying mutations in their NOD2 gene.

1.2 The genetic epidemiology of Crohn's disease

There is compelling epidemiological evidence including racial and ethnic differences in disease prevalence, and familial aggregation and twin studies suggesting a genetic contribution to the pathogenesis of Crohn's disease. Among ethnic groups, in the USA, the Ashkenazi Jewish population has the greatest risk of developing IBD compared with non-Jewish Caucasians, with an incidence rate 2-4 fold greater (Roth 1989). The lowest incidence and prevalence is found in Asian and African populations. (Bonen 2003)

Furthermore Crohn's disease is known to cluster within families. In population based studies it has been shown that the greatest risk factor for developing Crohn's disease is having other family members with the disease. Indeed both by case control and cohort studies the relative risk for Crohn's disease amongst first degree relatives is a 14 -15 fold

increased risk of developing CD (Orholm 1991, Satsangi 1996). Furthermore it has been reported that approximately 75% of multiply affected families with IBD are concordant for disease type and site of disease. (Binder 1998) This further supports the hypothesis of a genetic predisposition.

Disease aggregation in families may, however, be due to shared genetic and environmental factors. Twin concordance studies therefore, provide stronger evidence to evaluate genetic and environmental factors in Crohn's disease. Concordance amongst monozygotic twins for Crohn's disease ranges from 42% to 58%, whereas amongst dizygotic twins the concordance rate is not significantly different from that for all siblings - 7%. (Tysk 1988, Thompson 1996)

Collectively these findings lend compelling support to the inference that susceptibility is inherited and furthermore the absence of simple Mendelian inheritance suggests that multiple gene products contribute to a person's risk of CD.

Over the last two decades a wide variety of candidate genes have been studied and substantial progress in the search for genetic determinants of IBD susceptibility has been made. Using genetic linkage studies and candidate gene association studies at least nine IBD susceptibility loci have been identified in the search for genetic determinant of IBD susceptibility (Hugot 1996, Satsangi 1996, Cho 1998, Duerr 1998, Ma 1999, Rioux 2000, Duerr 2000). These have had varying degrees of replication and statistical support. Whereas some loci are specific to either Crohn's disease (IBD1 on chromosome 16) or ulcerative colitis (IBD2 on chromosome 12) others confer susceptibility to IBD overall (IBD3 chromosome 6) These findings lend support to the idea that CD and Ulcerative Colitis are polygenic disorders that share some but not all susceptibility loci. (Figure 1.1)

In the past year two new single nuclear polymorphisms have also been identified and shown to be associated with Crohn's Disease - IL23R and ATG16L1 (Duerr 2006 and Rioux 2007)

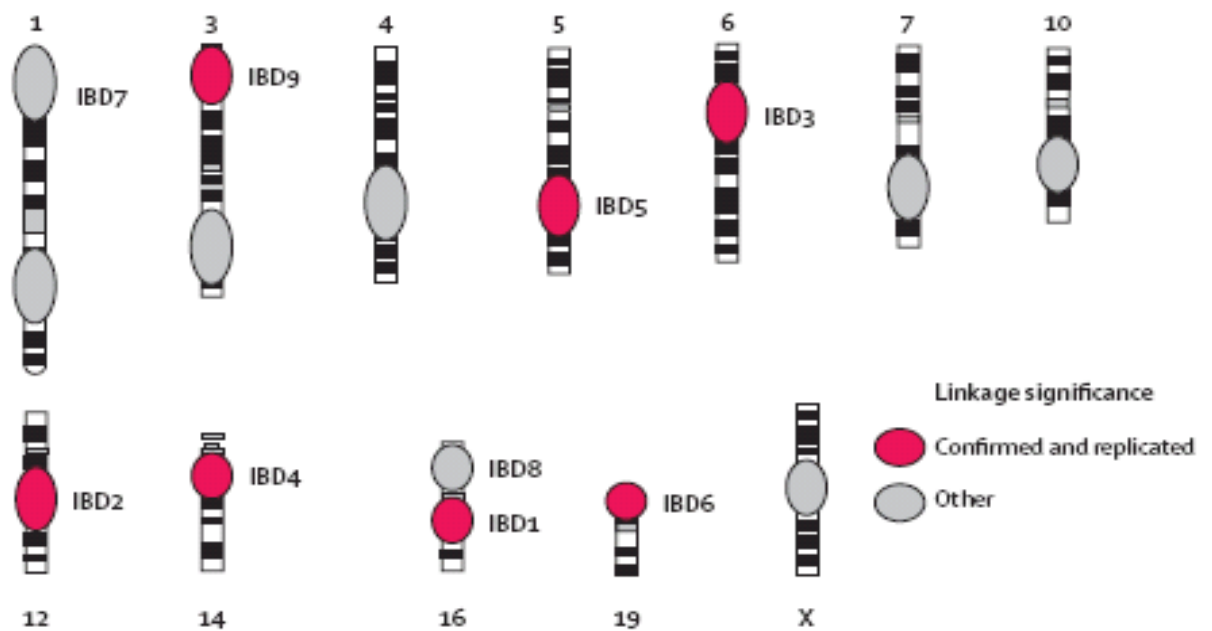


Figure 1.1 Susceptibility loci for inflammatory bowel disease as identified on genome scanning Figure adapted from Gaya et al 2006

1.2.1 IBD susceptibility loci - The IBD1 locus

In 1996 Hugot and colleagues were the first to identify a gene susceptibility locus in the pericentromeric region of Chromosome 16 which these investigators designated as IBD1. Widespread replication and confirmation of this linkage was soon obtained including pooled data from 12 centres in the International IBD Genetics Consortium study, which provided the strongest evidence for linkage with susceptibility to Crohn's Disease to this region. (Ohmen 1996, Brant 1998, Cavanaugh 1998, Curran 1998, Annese 1999, and Cavanaugh 2001) The evidence for linkage in this region is now known to be largely accounted for by the NOD2 gene. (See Chapter 1.4)

1.2.2 The IBD3 locus

The IBD3 locus on Chromosome 6p, encompassing the major histocompatibility complex has been consistently implicated for both Crohn's disease and Ulcerative Colitis in a number of linkage studies (Rioux 2000). Subtle changes within or near the peptide binding groove of the HLA class I and II peptides encoded within this locus lead to large interindividual differences in the capacity to respond to antigens in the acquired immune system. Both data from linkage and epidemiologic studies estimate the relative contribution of the HLA region to overall genetic risk as 10-33% for Crohn's disease. Interestingly recently the linkage seen at IBD3 has been suggested to be specific to men. (Fisher 2002) The specific risk allele or causative gene has thus far not been established.

1.2.3 The IBD5 locus

A region of significant linkage at chromosome 5q31-q33 was identified by Rioux (2001) and colleagues. This contributes to disease susceptibility in patients with early onset disease. This region contains a number of candidate genes encoding immunoregulatory cytokines IL-3, IL-4 and IL-5 that may be important in the pathogenesis of Crohn's disease. More recently there are data suggesting that polymorphisms in the organic cation transporter (OCTN) genes OCTN1 and OCTN2 represent disease-causing mutations within the IBD5 locus (Cho 2006). The causative role for these genes however is not yet proven.

1.3 Environmental factors in Crohn's disease pathogenesis

The fact that disease concordance is significantly less than 100% amongst monozygotic twins is the most powerful indicator supporting both a genetic and environmental factor in the pathogenesis of CD (Bonen 2003). Epidemiological and other evidence has identified a number of potentially important environmental factors.

1.3.1 Smoking

Of the potentially relevant environmental factors the best established is with tobacco exposure or smoking. Smokers are more than twice as likely to develop Crohn's disease as non smokers and smokers are also more likely to have ileal disease than ileocolonic or colonic (Loftus 2004). It is an unexplained but intriguing observation that only about 10% of patients with UC smoke, compared with 30% of the normal population and 40% of those with Crohn's disease (Timmer 1998). Indeed, in siblings, who have a similar genetic predisposition to IBD, those who smoke tend to develop Crohn's disease, while non-smokers get ulcerative colitis. (Bridger 2002) Smoking in Crohn's disease increases the risk of relapse, the need for immunosuppressive therapy and of surgery, especially in women (Cottone 1994). Furthermore, the natural history of Crohn's disease in patients who stop smoking is significantly better than in those who continue (Cosnes 2001). Interestingly there is no association between NOD2 risk allele carriage and smoking status. Multivariate analysis suggests that tobacco use, as well as NOD2 risk allele carriage are independent risk factors for developing ileal Crohn's disease. (Brant 2007)

1.3.2 Drugs

Relapse of IBD may be precipitated by non-steroidal anti-inflammatory drugs, (Bonner 2002), and by antibiotics, possibly secondary to adverse changes in enteric flora (Demling 1994). The latter conclusion is supported by case-control studies in adults and children showing an association between the onset of Crohn's disease and previous antibiotic usage (Card 2004). The oral contraceptive pill has also previously been associated

epidemiologically with Crohn's disease the risk being associated with duration of usage but not the oestrogen content of the preparation (Boyko 1994).

1.3.3 Diet

Because dietary antigens are, next to bacterial antigens, the most common type of luminal antigen, it is logical to surmise that diet may be important in the expression of IBD. Furthermore differences in diet may explain the differences in IBD incidence in migrant populations and across geographic regions. However despite numerous studies no consensus has emerged. Studies examining the association between diet and disease are difficult to perform due to the propensity for information bias. (Loftus 2004) However the most consistent association noted in dietary studies has been the link between high refined sugar and Crohn's disease. Other proposed dietary triggers include particles such as titanium oxide, aluminosilicates, (Powell 2000) fluoride and cornflakes, but none has yet been substantiated.

Patients with active Crohn's disease improve on replacement of ordinary food by a liquid formula diet (Fernandez-Benares 1994; Fernandez-Benares 1995; Griffiths 1995), and have been reported thereafter to deteriorate on introduction of specific foods (Riordan 1993).

In summary although no individual foods have been identified as universally detrimental to patients with Crohn's disease, it is conceivable that some foods may prove pathogenic, through direct adverse effects on mucosal immunity or by modifying luminal gut flora.

1.3.4 Bacteria

The role of bacteria in the pathogenesis of Crohn's disease is of fundamental and increasingly recognised importance. The normal human intestine is home to more than 400-500 species of bacteria and foreign antigens. Bacterial colonization is hindered in the stomach and proximal small intestine, due to the secretion of bile and acid and the normal intestinal motility. However the concentration of bacteria rises rapidly in the terminal ileum till in the colon it reaches 10^{10} to 10^{12} bacteria/g. This antigenic load is separated from the

gut associated lymphoid tissue (GALT) by only a single layer of intestinal epithelium. In health the mucosal immune system maintains active tolerance to commensal bacteria and food antigens whilst retaining the ability to respond to pathogens. In Crohn's disease this active tolerance is broken down and the inflammation is propagated by the intestinal flora. A role for the intraluminal bacteria in disease pathogenesis is suggested by the absence of inflammation in various genetic models of colitis when animals are raised in germ free conditions. (Fiocchi 1998) Furthermore antibiotic therapies are effective and extensively used in the treatment of Crohn's disease. (Hendrickson 2002)

1.3.4.1 Specific Pathogenic Bacteria

The literature contains many reports proposing an aetiological role for specific infectious agents in Crohn's disease but thus far, despite extensive research there is no compelling evidence of a single causative pathogen.

1.3.4.2 *Mycobacterium avium* subspecies *paratuberculosis* (MAP)

Over 20 years ago *Mycobacterium avium* subspecies *paratuberculosis* (MAP), a cell wall-deficient organism was first proposed as a causative agent for Crohn's disease after being isolated from CD tissue. MAP is the causative agent for Johne's disease a chronic granulomatous ileitis in cattle and sheep which clinically resembles CD.

The question has recently increased in intensity following the detection of the specific DNA insertion sequence, IS900 of MAP in 52% of patients with Crohn's disease compared to 2% of ulcerative colitis patients and 5% of normal controls (Autschbach 2007) Other arguments in favour of MAP being causative in Crohn's disease are reports of long term responses to antimycobacterial antibiotic combinations (Gui 1997) and also cultured viable *M. paratuberculosis* in blood samples of Crohn's disease patients. (Naser 2004) Furthermore different groups have detected MAP in both the food chain (Millar 1996) and water supply (Mishina 1996) increasing the possibility of MAP being causative in human CD.

However there are many flaws in the argument that MAP is pathogenic in CD as well as substantial evidence that it cannot be pathogenic. Firstly there is huge variability in detection of MAP by PCR and serological testing in CD patients ranging from 0-100% (Sartor 2005) and secondly genotypes of Crohn's disease MAP and bovine MAP are different (Motiwala 2003). Also importantly a cellular immune response to MAP has not been documented in CD patients despite increased serological responses to MAP in the same patients (Olsen 2001) Furthermore the observation that CD patients respond to chronic immunosuppressive therapies rather than have a worsening of their disease suggests MAP cannot be causative. Recently Selby and colleagues (Selby 2007) reported the results of combination antibiotic therapy for patients with Crohn's Disease and reported that there was no significant difference between treatment and placebo groups after 2 years again suggesting that MAP does not play a significant pathogenic role in CD.

In conclusion existing data do not compellingly implicate MAP as a causal agent in CD however neither does it definitively exclude the possibility. (Sartor 2005)

Two possible options to explain the discrepant findings are that MAP infection could cause CD in a subset of patients who are genetically susceptible to it or that alternatively this relatively common dietary organism may selectively colonise the ulcerated mucosa of CD patients but neither initiate or perpetuate the inflammation.

1.3.4.3 Adherent invasive *Escherichia coli* and *Yersinia*

Adherent invasive *Escherichia coli* have also been proposed as having a causative role in CD. Martin (Martin 2004) identified mucosa-associated *E.coli* in 43% and intramucosal *E.coli* in 29 % of CD compared to 17% and 9% respectively in control patients. *Yersinia enterocolitica* and *Y. pseudotuberculosis* have also been proposed in several studies as the cause of CD after being demonstrated in Crohn's disease lesions (Lamps 2003). Yersiniosis clinically resembles Crohn's disease making differentiating between these two conditions difficult. They both feature ileitis, ileocolitis, erythema nodosum and a reactive arthritis. Hugot (Hugot 2003) and colleagues proposed the 'cold-chain hypothesis' in 2003 after recognizing the rise of CD mirrors the rise of refrigeration. The cold chain hypothesis suggests that psychrotrophic bacteria such as *Yersinia* and *Listeria* species which are

capable of growing at low temperatures contribute to the disease. This has yet to be substantiated.

1.3.4.4 Measles

The association between both measles infection and measles vaccine and the development of IBD has received much attention in recent years, both in the scientific and popular press. Two systematic reviews (Robertson 2001, Davis 2001) however show no positive association.

1.3.4.5 Non-pathogenic bacteria

Recently the focus of research has changed from infectious to commensal bacteria. There is a substantial body of evidence demonstrating that the normal enteric flora plays a key role in the development of CD and the search is now on for alterations in the endogenous microflora which may be involved in initiating or perpetuating inflammation in CD. The most convincing data of the importance of gut flora comes from animal models. Taurog and colleagues (Taurog 1994) demonstrated that intestinal inflammation fails to develop where transgenic rats are kept in a germ free environment though once introduced to normal flora colitis rapidly developed. Furthermore the diversion of faecal stream from inflamed bowel loops is known to induce symptomatic improvement in CD whilst restoring intestinal continuity can trigger a recurrence.(D'Haens 1998) Studies of animal models resonate with clinical experience. Broad spectrum antibiotics have proven efficacy and are used regularly in specific subgroups of patients.

Furthermore changes in the normal bacterial flora have been demonstrated in CD. (Fabia 1993, Swidsinski 2002) Fabia et al reported radically reduced concentrations of anaerobic bacteria and *Lactobacillus* in active IBD pts whilst Seksik (Seksik 2003) reported that the faecal microflora in patients with both active and inactive colonic Crohn's disease differed from the faecal microflora in healthy controls. The two main differences they demonstrated were that the patients harboured significantly more enterobacteria and that 30% of their faecal bacteria did not belong to the usual dominant groups found in the healthy controls. (Seksik 2003) Other studies have demonstrated the presence of an increased number of

surface-adherent and intra- cellular bacteria in the colonic epithelium of patients with IBD.(Swidsinski 2002, Darfeuille-Michaud 1998) It has also interestingly been demonstrated that different bacteria illicit different cytokine responses from immune or intestinal cells. (Borruel 2003)

Furthermore recently probiotics which are live, non-pathogenic bacteria that confer health benefits beyond their nutritional value have been used in the experimental treatment of CD. Currently the beneficial effect of probiotics is demonstrated mainly in pouchitis and ulcerative colitis though their efficacy proves the importance of the role of the gut flora. Gionchetti (2003) demonstrated that the probiotic VSL#3 was effective in preventing acute pouchitis in patients who underwent ileo-anal pouch formation in UC having previously reported its use in preventing relapses in chronic relapsing pouchitis. Conversely Prantera (2002) demonstrated no benefit from *Lactobacillus rhamnosus* CG in preventing endoscopic recurrence of CD after surgery. These observations therefore support the importance of the role of the gut flora without allowing wide extrapolation. (Marteau 2004) In the future it is hopeful that identifying variations in the flora may help in developing new treatment strategies.

Finally the role of bacteria in the pathogenesis of CD is supported by the finding of increased intestinal permeability in patients with CD (May 1993). Increased intestinal permeability has been suggested as an early predisposing factor which may precede the development of clinical disease. (Schmitz 1999) Recent data reporting an association between genes important in mucosal transport and integrity (OCTN & DLG5) further support this association

1.4 Identification of the NOD2 gene

In 1996, Hugot and colleagues, using genetic linkage studies, identified a gene locus on chromosome 16q12, named IBD1, which is specifically associated with Crohn's disease (Hugot 1996). In 2001, two groups of investigators working independently, identified the NOD2 gene within the IBD1 gene locus, and showed that mutations of the NOD2 gene were strongly associated with Crohn's disease (Hugot 2001, Ogura 2001). Hugot and colleagues (2001) screened 235 Crohn's disease families to identify the NOD2 gene on the

IBD1 locus using a positional-cloning strategy, based on linkage analysis followed by linkage disequilibrium mapping, then on pedigree disequilibrium mapping, and finally on extensive sequence analysis of the best candidate gene. Three independent associations with Crohn's disease, a frameshift variant and two missense variants of NOD2 were identified by this approach. Ogura and colleagues (2001), alternatively identified the NOD2 gene whilst investigating apoptotic proteins, and intuitively considered NOD2 a candidate gene on the IBD1 locus as this gene is located in the peak region of linkage on chromosome 16. Using the transmission disequilibrium test and case-control analysis, these investigators identified a frameshift mutation that is associated with Crohn's disease (Ogura 2001)

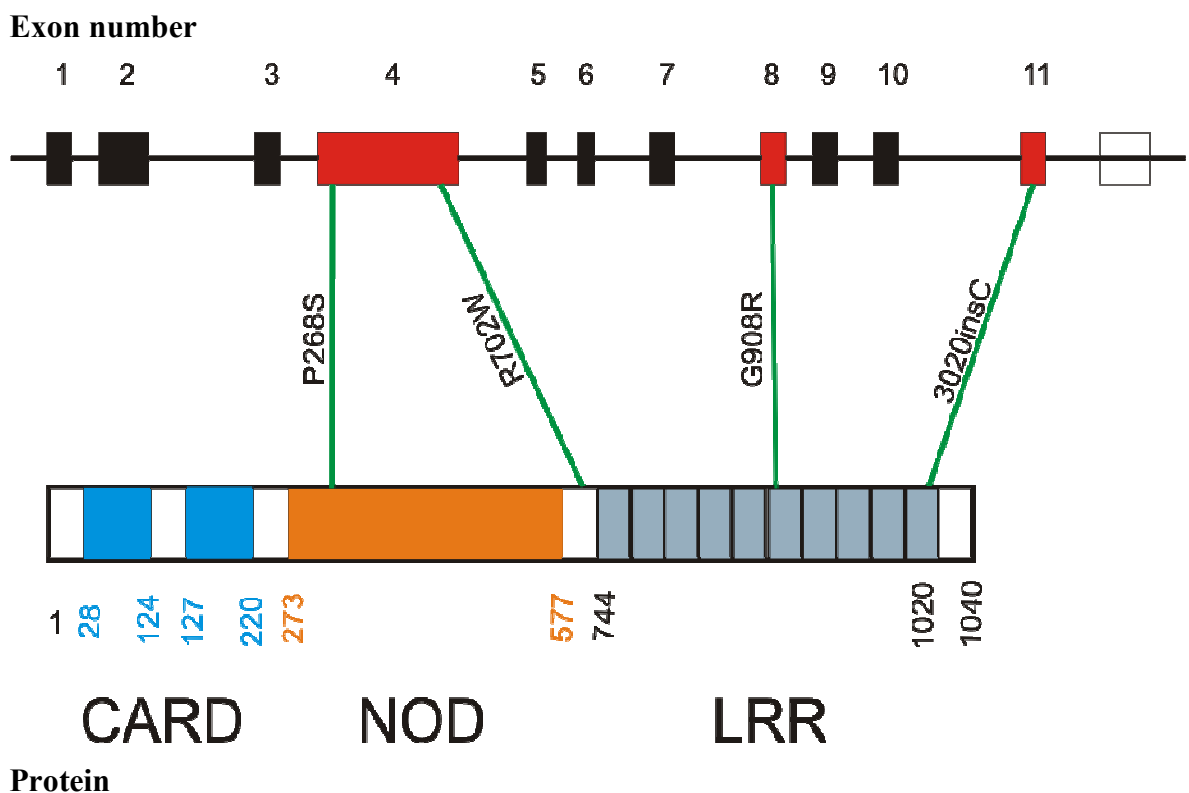


Figure 1.2 The genomic structure of the NOD2/CARD 15 gene, showing the location of Crohn's disease-associated variants, and predicted protein structural domains. The three major coding region polymorphisms Arg702Trp (R702W), Gly908Arg (G908R), and Leu1007fsinsC (3020insC) are indicated. All three common variants occur together with a background mutation, Pro268Ser (P268S). CARD: Caspase-recruitment domains; NOD: Nucleotide-binding domain; LRR: Leucine rich repeats.

1.4.1 NOD2 Mutations associated with Crohn's disease

There are three common genetic variants of the NOD2 gene that are associated with Crohn's disease. The frameshift mutation, identified by both Hugot (2001) and Ogura (2001), is caused by a single cytosine base insertion in exon 11 at nucleotide position 3020 (3020insC), which results in a frameshift at the second nucleotide of codon 1007, and substitutes proline for leucine in the tenth LRR, followed by a premature stop codon. Hugot and colleagues (2001) identified two other single nucleotide polymorphisms (SNP), designated with the marker names SNP8 and SNP 12 which are associated with Crohn's disease. SNP8 is a point mutation, substituting Cytosine for Tryptophan, at nucleotide position 2104 and SNP12 is a point mutation, substituting Guanine for Cytosine, at nucleotide position 2722. These 2 mutations encode for amino acid substitutions, tryptophan substituted for arginine at codon 702 (Arg702Trp) and arginine substituted for glycine at codon 908 (Gly908Arg). These three major mutations all occur within or near the leucine-rich repeats (LRR)-encoding region of the gene (Figure 1.2). All three common variants occur together with a background mutation, Pro268Ser (serine substituted for proline at codon 268). Hugot and colleagues (2001) also described over 30 other much rarer amino acid polymorphisms occurring near or within the LRR-encoding region of the gene.

Subsequently, many studies have consistently shown the presence of higher allelic frequencies for Arg702Trp, Gly908Arg, and Leu1007fsinsC in Caucasian Crohn's disease-affected patients. Economou et al (Economou 2004) published a large meta analysis of these studies assessing NOD2 polymorphisms associations with CD and UC. Forty –two studies were included and the findings are summarised in Figure 1.3a. The strongest association is found with Leu1007fsinsC although homozygous carriage of this mutation does not always lead to IBD. (Linde 2003) Overall, approximately 8% to 17% of Crohn's disease-affected patients carry two copies of the major NOD2 risk alleles compared to less than 1% of the control Caucasian population. About 27% to 32% of Caucasians patients carry one copy of these risk alleles compared with about 20% of controls. The 3 risk alleles occur in the healthy population in varying frequencies. The genetic association between the rarer polymorphisms and Crohn's disease is difficult to ascertain due to the rare occurrence of these mutations. In summary heterozygous carriage of a NOD2 risk allele increases risk

of developing CD 2-4 fold whereas double dose carriage increases risk 20-40 fold. (Cuthbert 2002, Cavanaugh 2002).

Figure 1.3a

Association of individual NOD2 SNPs with Crohn's Disease expressed as odds ratios

	SNP8 OR (95%CI)	SNP12 OR (95%CI)	SNP13 OR (95%CI)
Non-Jewish Caucasians	2.20 (1.84-2.62)	2.99 (2.38-3.74)	4.09 (3.23-5.18)
Jewish Caucasians	1.74 (0.88-3.42)	1.93 (1.23-3.00)	2.45 (1.51-3.98)
Asians	Not found	Not found	Only found in one study*

* Guo *et al*, 2004. SNP13 allele frequencies: CD 3.3%, UC 1.3%, controls 0.3%, OR – odds ratio, (adapted from Economou *et al*, 2004).

Figure 1.3b

Carriage of NOD2 variant SNP and association with Crohn's Disease phenotype

Variable	OR (95%CI)
<i>Dose-response relationship</i>	
Carriage of one allele	2.39 (2.00-2.86)
Carriage of two alleles	17.1 (10.7-27.2)
<i>CD phenotype</i>	
Familial vs sporadic	1.49 (1.18-1.87)
Small bowel affected vs not affected	2.53 (2.01-3.16)
Stricture/stenosing vs other behaviour	1.94 (1.61-2.34)
Colonic CD only vs healthy controls	1.41 (1.71-1.69)

OR- odds ratio, adapted from Economou *et al*, 2004

1.4.2 Racial and geographical differences in NOD2 genotype expression

Investigations into the inheritance of the three common disease causing risk alleles Arg702Trp, Gly908Arg and Leu1007fsinsC in NOD2 associated with susceptibility to CD have demonstrated a remarkable amount of heterogeneity across ethnicities and populations with even regional differences across Europe. Several authors have previously commented on evidence for a North-South gradient in CD in Europe with a higher incidence in Northern Europe however the frequencies of the NOD2 risk alleles do not underlie this gradient. Arnott (2004) reported lower frequencies of the NOD2 risk alleles in Scottish, Irish & Scandinavian CD patients than in English, Central Europe or North American patients and Vind (2005) compared Danish & Portuguese patients and controls and again found no evidence to support this hypothesis.

Increasing evidence shows the highest frequencies of the risk alleles in central and Southern Europe. In a comparison of NOD2 allele frequencies in 3575 Caucasian healthy individuals from 16 different centres across Europe, North America and Australia Hugot (2007) showed the lowest frequencies of the known variants in Australian and Finland populations whereas Belgium and Canada had the highest frequency of the known NOD2 risk alleles.

In specific non-Caucasian racial populations, the common NOD2 mutations show no significant contribution to CD susceptibility. A study by Inoue (2002) showed there were no carriers in Japanese and Korean populations for any of the 3 common NOD2 risk alleles seen in Caucasian populations amongst 350 CD patients or 292 controls. More recently the NOD2 risk alleles have also been found to be absent in the Han-Chinese population.

Furthermore carriage of the 3 major NOD2 risk alleles is considerably lower amongst African-American and Hispanic Crohn's patients compared with Caucasian CD patients or controls. (Cavanaugh 2006) Little information is available from African or South American populations with the exception of a recent report of low NOD2 allele frequencies in coloured South Africans of Indian descent. (Cavanaugh 2006)

Amongst Caucasians Bonen (2003) reported comparable allele frequencies of the NOD2 frameshift mutation Leu1007fsinsC in both Jewish (7.3%) and non-Jewish (8.4%) Crohn's

patients whereas the allele frequency of G908R was significantly higher among Ashkenazi Jews (10.2%) compared with non-Jewish CD patients (4.3%). This association has been confirmed in other studies.

In summary the 3 major risk alleles are found primarily in Caucasian CD and in some populations the NOD2 risk allele frequencies are high – in up to 50% of CD patients. However the incidence of CD in populations with high carrier frequencies of the NOD2 risk alleles for example Belgium and Canada is remarkably similar to those with lower rates suggesting the presence of other genetic susceptibilities.

1.4.3 NOD2 genotype and Crohn's disease phenotype

Crohn's disease, a complex heterogeneous disorder, is classified all be it imperfectly according to the Vienna classification. The Vienna Classification was developed by an international working party in 1996 and classifies patients according to there categories; age of diagnosis, location of disease and disease behaviour. (Appendix B) Age at diagnosis is divided into <40 years (A1) and over 40 years (A2) Location of disease is subdivided in to terminal ileum (L1), colon (L2), ileocolon (L3) and upper gastrointestinal tract (L4). These subgroups apply to the maximal extent of disease prior to the first surgery. Disease location is moderately stable in the short term but after 10 years there can be significant changes (Louis 2001). Finally disease behaviour is divided into inflammatory (B1), stricturing (B2) and penetrating (B3). It is disease behaviour which is the most contentious of the categories as it has been shown that 46% of patients initially diagnosed with inflammatory (B1) disease phenotype will go onto develop stricturing or penetrating (B2 or B3) within 10 years. (Louis 2001) Accepting these restrictions numerous investigators have sought to determine the association between NOD2 genotype and Crohn's disease phenotype.

1.4.3.1 Disease location

Numerous studies have established that mutant NOD2 risk allele carriage is specifically associated with ileal disease (Ahmad 2002, Cuthbert 2002, Lesage 2002). In European patients, the frequency of the NOD2 risk alleles is significantly higher in patients with exclusive ileal disease (26.9%), intermediate in those with ileocolitis (19.7%), and lowest in those with colonic disease (12.7%) (Cuthbert 2002). Lesage and colleagues (2002) have reported that in French patients with Crohn's disease, colonic involvement is significantly less common in individuals carrying two copies of the risk NOD2 alleles than in individuals carrying wild-type alleles.

This site specific association has also been confirmed in an analysis of American patients with Crohn's disease (Brant 2003). Using logistic regression analysis, these investigators determined that carriage of two NOD2 risk alleles, either homozygous or compound heterozygous was the strongest predictor of ileal disease. NOD2 mutations were associated with a 10-fold risk of developing ileal disease, even after adjusting for the risks associated with cigarette smoking and age at diagnosis.

Other studies have also confirmed that the NOD2 variant alleles specifically confer risk for ileal disease. In a recent meta-analysis, Economou and colleagues (2004) assessed the effects of NOD2 variants alleles on Crohn's disease phenotype in non-Jewish Caucasian populations, and showed that the strongest genetic effect of NOD2 mutations was predisposition to small bowel disease. This meta-analysis confirmed that the presence of a NOD2 risk allele does not confer any increased risk for Crohn's disease when cases with only colonic disease are considered. Figure 1.3b

1.4.3.2 Age at diagnosis

Presence of the NOD2 risk alleles is associated with an earlier age of diagnosis in Crohn's disease. The disorder is diagnosed on average 3-5 years earlier in European and American patients who are homozygous or compound heterozygotes for the NOD2 mutations. (Economou 2004)

1.4.3.3 Disease behaviour

Disease behaviour is the most difficult to study, because as stated previously, it changes over time, and a longer duration of inflammation makes it more likely that stricturing or penetrating complications will develop in an individual with Crohn's disease. Given this caveat, some studies have shown an association between NOD2 variant allele carriage and both stricturing and penetrating phenotype. (Lesage 2002, Brant 2003, Heliö 2003) One meta-analysis of 13 studies showed that the presence of at least one high-risk variant allele increases the risk of stricturing disease 1.9 fold. (Economou 2004)

1.4.3.4 Surgery and Infliximab

Most studies have also shown that in adults the NOD2 variant haplotype does not influence the need for surgery independently of disease location. (Economou 2004) However in children carriage of NOD2 mutations does confer an increased risk of need for surgery. (Gaya 2006). Thus far pharmacogenetic studies assessing the usefulness of NOD2 mutations in predicting outcome of treatment with infliximab (anti TNF antibody) have found no association between predictive response from infliximab treatment and mutant NOD2 carriage.

In conclusion, patients carrying the NOD2 mutations are more likely to have early onset disease, terminal ileal involvement, and stricturing disease, all phenotypic characteristics initially implicated in Crohn's initial description of regional enteritis. (Crohn 1932)

1.4.4 NOD2 mutations associated with other diseases

Since the association between NOD2 mutations and Crohn's disease was identified, other diseases have been associated with mutations in NOD2. The first non-IBD association was with a rare disease long known to map to the pericentromeric region of chromosome 16 - Blau Syndrome. Blau Syndrome is an autosomal-dominant disorder characterised by skin rashes, uveitis and arthritis with granulomatous inflammation. In contrast to Crohn's Disease mutations that confer susceptibility to Blau syndrome occur in the NOD encoding

region of the NOD2 gene as opposed to the LRR domain linked to CD. (Miceli-Richard 2001) Interestingly Early Onset Sarcoid which clinically resembles Blau Syndrome has also been recently demonstrated to be associated with NOD2 mutations (Kanazawa 2005)

The second association identified is with gastrointestinal graft-versus-host disease. Holler and colleagues (2004) analysed NOD2 mutations in a 169 recipients of allogeneic blood stem cell transplantation and their respective donors. The presence of NOD2 mutations in either donor or host was associated with a significantly increased frequency of intestinal graft-versus-host disease and mortality. Where both donor and recipient carried the NOD2 mutations mortality was increased from 24% to 60%. The authors speculate that the NOD2 mutations impair the microbial response of the gastrointestinal epithelium resulting in septicaemia and multi-organ failure.

There are reports suggesting an association between carcinoma and NOD2 mutations. Kurzawski (2004) reported that an association between the Leu1007fsinsC mutation and colorectal cancer in a Polish population, and more recently there has been a suggestion that the NOD2 risk alleles may be involved in breast cancer in individuals with first degree relatives with lung cancer. (Lener 2006) These studies require further validation before an association can be confirmed.

A number of autoimmune diseases, for example rheumatoid arthritis and systemic lupus erythematosus, have been shown to demonstrate linkage to the pericentromeric region of chromosome 16 that includes NOD2 and as a result various investigators have tested specific diseases for the common NOD2 mutations (Cavanaugh 2006). Thus far published reports suggest that there is no association between NOD2 gene mutations and rheumatoid arthritis (Steer 2003), systemic lupus erythematosus (Ferreiros-Vidal 2003), Wegener's granulomatosis (Newman 2003), Behcet's disease (Uyar 2004) or sarcoidosis (Martin 2003) With regard to infectious diseases, no association has been identified between NOD2 gene mutations and pulmonary tuberculosis (Stockton 2004) and periodontitis (Folwaczny 2004, Laine 2004),

1.5 NOD2 Protein

1.5.1 The structure of the NOD2 protein

The NOD2 protein, comprising of 1040 amino acids, is composed of two N-terminal caspase-recruitment domains (CARD) (residues 28-220) fused to a centrally located nucleotide binding domain (NBD) (residues 273-577) containing consensus nucleotide-binding motifs followed by 10 tandem leucine-rich repeats (LRRs) (residues 744-1020) (Figure 1.2). The frameshift mutation Leu1007fsinsC encodes for a truncated NOD2 protein containing 1007 amino acids, instead of 1040.

The NOD2 protein is a member of a large family of intracellular proteins – the NOD (nucleotide-binding oligomerization domain) proteins- that are widely distributed in nature and includes the resistance (R) proteins in plants that are involved in plant host defence against pathogens. (Ogura 2001) Thus far over 20 NOD containing proteins have been identified in humans, nematodes and plants (Inohara 2005). Some of these proteins are shown in Figure 1.4. This family of proteins is alternatively classified as the CATERPILLER family of proteins– a family of proteins that are implicated in intracellular recognition of bacterial components (Inohara 2002 & Girardin 2003). CATERPILLER is an acronym for CARD, transcription enhancer, R (purine)-binding, pyrin, lots of leucine repeats (Ting 2005). The family also includes the protein NOD1. NOD1 is NOD2's closest relative in mammals and is a ubiquitously expressed protein having a similar structure to NOD2 except for a single CARD domain compared to NOD2's two.

The majority of animal and plant NOD proteins are comprised of three distinct domains: an amino-terminal effector domain, a centrally located regulatory NOD domain, and a carboxyl-terminal LRR domain. Structurally the NOD2 protein consists of two amino terminal effector domains, known as caspase-recruitment domains (CARD) which mediate protein-protein interactions, fused to a centrally located nucleotide binding domain (NBD) containing consensus nucleotide-binding motifs which mediate self – oligomerization required for activation, followed by 10 tandem leucine-rich repeats (LRRs). The LRR domain is of particular importance as the three common coding region polymorphisms

Arg702Trp, Gly908Arg and Leu1007fsinsC associated with Crohn's disease occur within this domain.

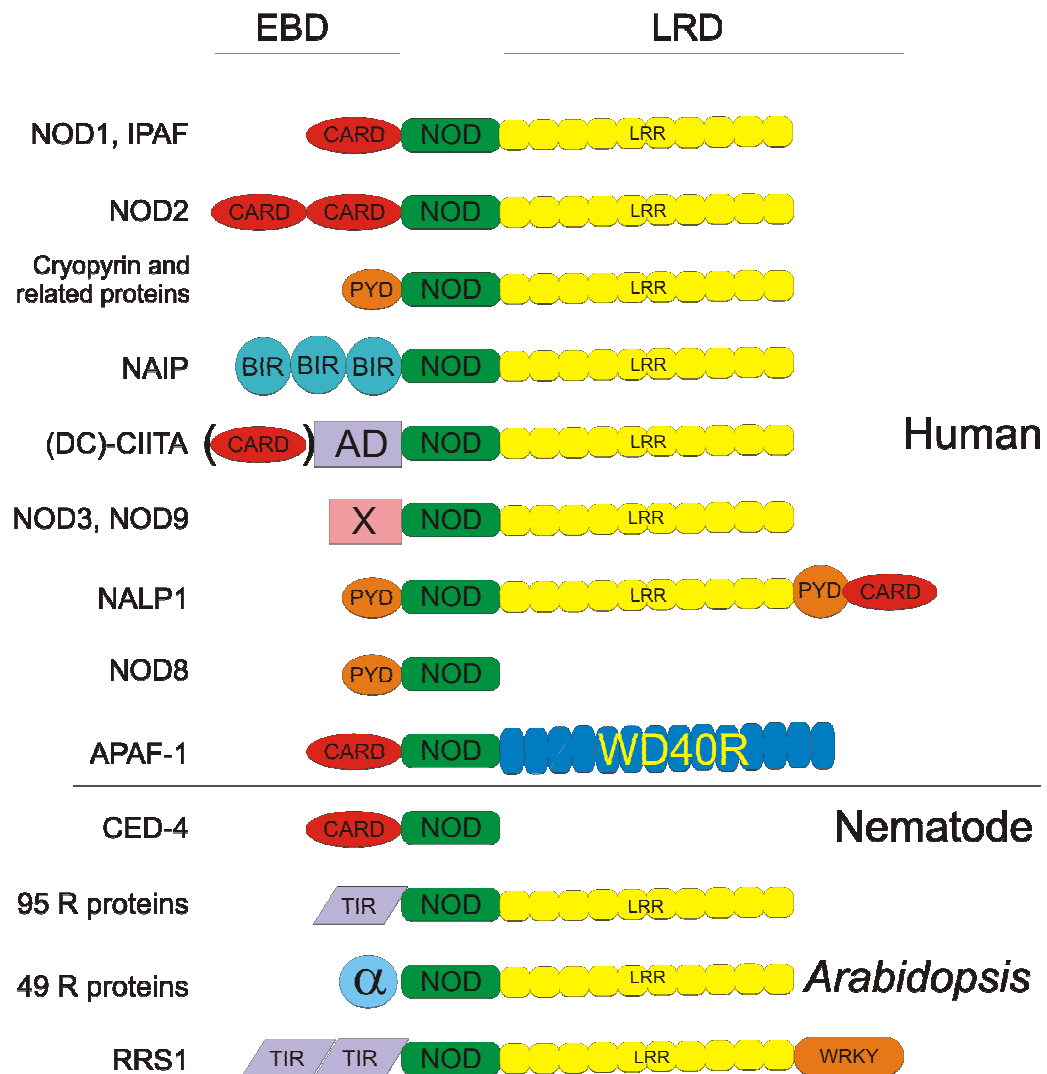


Figure 1.4 The domain structure of the NOD proteins found in humans, nematodes and Arabidopsis (plants) share structural homology. Most NOD proteins are composed of variable amino-terminal effector-binding domains (EBD), a centrally located nucleotide-binding oligomerization domain (NOD) that mediates self-oligomerization, and a carboxyl-terminal ligand-recognition domain (LRD). The number of leucine-rich repeats (LRRs) varies in NOD proteins. Other abbreviations: CARD - caspase-recruitment domain, PYD - pyrin domain, BIR-baculoviral inhibitor-of-apoptosis repeat, TIR - Toll interleukin-1 receptor domain, Apaf-1 - apoptotic protease activating factor 1. *Figure adapted from Inohara (2005)*

1.5.1.1 CARD domain

The CARD domains of NOD2 share significant sequence similarity with CARD motifs found in a variety of apoptotic signalling molecules, such as CED-4 and APAF-1 (Bertin 1999). The CARD domain is a protein fold consisting of a tightly packed, six-membered α -helical bundle. The CARD motif functions as effector domains that mediate specific homophilic interactions with down-stream CARD-containing signalling molecules. It is believed that proteins with CARD domains are centrally involved in assembling protein complexes that drive activation of either caspases or I κ B kinase (IKK) by facilitating close proximity of the latter molecules (Martin 2001). Both the NOD proteins, NOD1 and NOD2, interact with RICK through homophilic CARD-CARD interactions. RICK associates with the γ subunit of I κ B kinase (IKK γ) that complexes with IKK α and IKK β to then induce the degradation of I κ B which, subsequently results in NF- κ B activation (Inohara 2000).

1.5.1.2 NOD domain

The centrally located nucleotide oligomerization domain (NOD) of NOD2 mediates self-oligomerization leading to NF- κ B activation. (Abraham 2006) The NOD region has residues at catalytic sites which are essential for binding and hydrolysis of Mg²⁺-ATP or –GTP, and are predicted to share common nucleotide binding folds with ATP-binding cassettes (ABC) of members of the ABC ATPase/GTPase superfamily (Inohara 2000). Point mutations in the phosphate chain binding site (known as P-loop) are predicted to result in loss-of-function of NOD2, since corresponding point mutations in NOD1 P-loop result in loss-of-function. (Inohara 1999)

1.5.1.3 LRR domain

The LRR domain of NOD2 is similar to the LRR of Toll like receptors which mediate recognition of pathogen-associated molecular patterns (PAMP). Each of the NOD2 LRR's have structural homology to LRR's of the plant disease resistance (R) proteins, and each

LRR contains a predicted α helix and β sheet sequence that is consistent with the prototypical horseshoe-shaped structure of R protein LRR's (Ogura 2003). The LRR's of these proteins recognise distinct effector molecules from pathogenic bacteria, and it is currently suggested that the NOD2 LRR's perform a similar function. The NOD- and LRR-containing proteins, however, are relatively abundant and functionally diverse in mammalian cells, and an interaction between the LRR's and bacterial products in mammalian cells has thus far not been proven (Beutler 2001).

1.5.2 NOD2 Protein Expression

1.5.2.1 Monocytes

The original expression studies by Ogura determined the tissue expression of NOD2 by northern blot analysis. They localised NOD2 expression to peripheral blood leukocytes, with little or no detectable expression in various other human tissues. This compared to NOD1 which is present in most tissues. They further fractionated peripheral blood leukocytes into its granulocytic, monocytic and lymphocytic components and established, that NOD2 expression was most abundant in monocytes (Ogura 2001a) Gutierrez went on to demonstrate by quantitative real-time PCR analysis that NOD2 was expressed in monocytes but also granulocytes and dendritic cells and to a lesser extent in T lymphocytes. (Gutierrez 2002).

1.5.2.2 Epithelial Cells

Following the discovery that it is expressed constitutively in myeloid cells other investigators subsequently showed that NOD2 mRNA was also expressed in intestinal epithelial cells. (Rosenstiel 2003) NOD2 mRNA and protein expression was upregulated in intestinal epithelial cells and in primary colonic epithelial cells by TNF α and γ IFN. (Rosenstiel 2003)

1.5.2.3 Paneth Cells

Using in situ hybridisation, immunohistochemistry and laser capture microdissection Lala et al demonstrated that NOD2 mRNA and protein is expressed in Paneth cells in the small intestine with the greatest intensity being noted in the terminal ileum where Paneth cells are most numerous. NOD2 expression was increased in Paneth Cells from patients with inflammatory bowel disease both in the terminal ileum and due to the presence of metaplastic Paneth cells in the colon (Lala 2003, Ogura 2003(a)) (See Section 1.6)

1.5.3 NOD2 protein Function –PAMP recognition

It was originally proposed that the NOD2 protein was an intracellular receptor for bacterial products, in particular LPS. This hypothesis was correlated with two important findings. Toll like receptors also contain LRR's and are linked to the recognition of pathogen derived products, including LPS. Secondly NOD proteins have domains similar to those of plant R proteins which are involved in resistance to pathogens therefore the NODs were thought to be responsible for the intracellular discrimination of pathogen products, analogous to the extracellular recognition functions mediated through the TLR's. The intracellular recognition of LPS by the NODs was thought to activate NF κ B and other critical innate defence mechanisms.

1.5.3.1 Initial Hypothesis of NOD2 function – Receptor for LPS

Inohara and colleagues (2001a) initially showed that in NOD2-expressing transfectants, NF- κ B activation increased in response to LPS stimulation and, to a lesser extent, to PGN stimulation (Inohara 2001a). Furthermore they suggested that the LRR motifs was essential for enabling the NOD2 protein to respond to LPS, which was consistent with the prevailing view that LPS interacts with the LRR domains of R proteins and TLR with subsequent NF- κ B activation. (Inohara 2001a) Interestingly, in mammalian cells transfected with an expression plasmid encoding the mutant Leu1007fsinsC-NOD2 protein, LPS induced less NF- κ B activation and it was therefore postulated that mutant NOD2 proteins fail to mediate

cellular activation in response to LPS (Ogura 2001). Based on these findings, it was widely accepted at the time that NOD2 was an intracellular receptor for LPS (Hugot 2001).

NOD2 was thought to be responsible for the intracellular discrimination of pathogens, analogous to the extracellular recognition functions mediated through the TLR's.

1.5.3.1.1 Toll like Receptors

Toll like receptors (TLR's) are a family of pattern recognition receptors that sense motifs found in microbial pathogens called pathogen associated molecular patterns (PAMPS). TLR's detect microbial infection and initiate immune defence against pathogens. The majority of TLR's are located on the cell surface. This is opposed to NOD2 which is expressed intracellularly, within the cytoplasm. Thirteen TLR's named simply TLR1 to TLR13 have been identified in humans and each has a specific well conserved ligand. The recognition of PAMPS by toll like receptors leads to the activation of the NF- κ B signalling pathway.

1.5.3.2 NOD2 recognises bacterial Muramyl dipeptide (MDP)

Bonen and colleagues (2003a) using HEK293T transfectants, first showed that peptidoglycan (PGN), mediated NOD2-induced cellular activation. Later, two independent groups of investigators showed that a component of bacterial PGN, called muramyl dipeptide (MDP) is the minimal component which induces NOD2-mediated cellular activation (Girardin 2003, Inohara 2003).

Girardin and colleagues (2003) showed that purified PGN activates NF- κ B in mammalian cells that were simultaneously transfected with NOD2 expression plasmids. They subsequently determined that cellular activation mediated by NOD2 was due to MDP, a component of gram-positive and gram-negative bacteria. Using chemically synthesised MDP they verified that MDP mediates NOD2-induced NF- κ B activation. As MDP did not induce cellular activation in cells expressing NOD1, TLR1, TLR2 and TLR6, Girardin and

colleagues (2003) described NOD2 as the first protein that mediates cellular activation in response to MDP.

At the same time Inohara and colleagues (2003) using biochemical and functional analyses also went on to characterise the bacterial moiety that interacts with NOD2. Their studies also demonstrated & confirmed that only MDP specifically induces NOD2-mediated NF- κ B activation. Once activated by MDP, NOD2 activates downstream inflammatory signalling pathway through RICK. Furthermore they showed that NOD2 mutants associated with susceptibility to CD were deficient in their recognition of MDP and particularly those homozygous for the frameshift mutation.

1.5.3.2.1 The structure of peptidoglycan (PGN) and muramyl dipeptide (MDP)

Peptidoglycan (PGN) is an essential and unique component of bacteria that provides rigidity and structure to the bacterial cell wall. Virtually all bacteria contain a layer of PGN but the amount, location and specific composition vary. (McDonald 2005) In gram positive bacteria the PGN layer is a thick layer and found in association with lipoteichoic acid (LTA) whereas in gram negative bacteria it is a thin layer and overlaid by a thick layer of Lipopolysaccharide (LPS). PGN is composed of carbohydrate chains of β (1-4)linked, alternating N-acetylglucosamine and N-acetylmuramic acid sugars crosslinked by short peptide chains with alternating L and D amino acids. Two major types of PGN can be classified by the nature of the third residue of the stem peptide. In Gram negative bacteria it is commonly lysine whereas in Gram positive bacteria it is classically diaminopimelic acid (mesoDAP) however there is considerable variation. See Figure 1.5

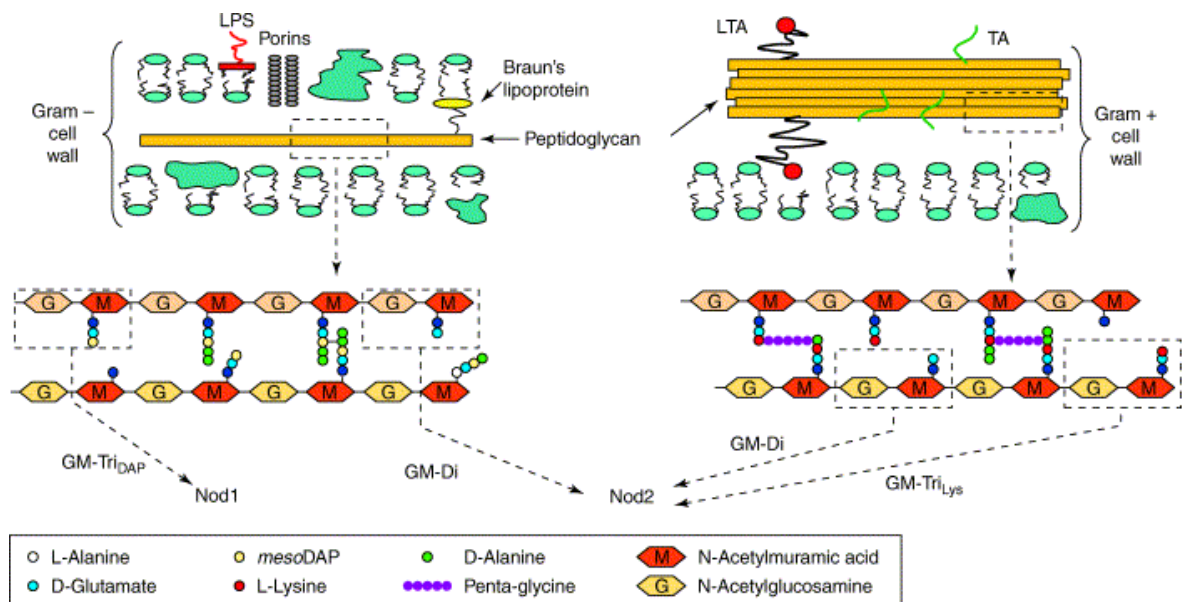


Figure 1.5 The structure of peptidoglycan showing the muropeptides that are postulated to interact with NOD2. Abbreviations: LTA, lipoteichoic acid; LPS, lipopolysaccharide; TA, teichoic acid. Figure from Girardin (2003a)

In gram negative bacteria there is a thin layer of peptidoglycan in the periplasmic space (left). Cross-linking of two parallel glycan strands through stem peptides occurs at low frequency. This cross-linking is made by a direct link between a *meso*DAP amino acid and the D-Alanine in position four from a peptide anchored on a parallel glycan strand. In gram-positive bacteria, peptidoglycan represents a thick structure that surrounds the bacterial membrane (right). Glycan strands are generally cross-linked to a high degree. In contrast to gram-negative bacteria, the cross-linking requires additional bridging amino acids, for example, through penta-glycine bridges, which are a characteristic of *Staphylococcus aureus* peptidoglycan (shown in the figure). NOD2 ‘senses’ two types of muropeptides: GM-Di (GlcNAc-MurNAc-L-Ala-D-Glu, also known as GMDP) present in peptidoglycans from gram-negative and gram-positive bacteria, and GM-Tri_{Lys} (GlcNAc-MurNAc-L-Ala-D-Glu-L-Lys), a muropeptide found only in peptidoglycans from gram-positive bacteria.

1.5.3.3 NOD2 protein Function - Signalling pathways induced

1.5.3.3.1 NOD2 induces NF- κ B activation

Initially it was presumed NOD2 would be involved in apoptosis but it has since been established that NOD2 associates through its CARD domains with the RICK protein kinase leading to activation of NF κ B and subsequent induction of genes encoding pro-inflammatory cytokines. Figure 1.6

The transcription factor nuclear factor- κ B (NF- κ B) is present in the cytoplasm in an inactive form, bound to the inhibitory protein I κ B (or inhibitor of NF- κ B). Phosphorylation of I κ B, mediated by I κ B kinase (IKK) a complex of IKK α , IKK β , and IKK γ results in degradation of I κ B, and translocation of NF- κ B to the nucleus where it induces gene transcription (Li 2002). NOD2-induced NF- κ B activation was inhibited in mammalian cells expressing mutant forms of IKK α , IKK β , IKK γ , and I κ B α which indicated that NOD2 acts upstream of the IKK complex. This finding was verified by expression studies (Yamaoka 1998, Inohara 2000).

Further analysis revealed that NOD2 interacts with the RICK protein through its CARD domain, upstream of the IKK complex, before inducing NF- κ B activation (Inohara 2000). Firstly, NOD2-induced NF- κ B activation was inhibited in cells expressing mutant RICK proteins. Then, the association between NOD2 and RICK proteins was proven in a series of transfection experiments using plasmids expressing wild-type or mutant forms of NOD2 and RICK. The association was only evident between native RICK and wild-type or mutant NOD2 protein that contained both CARD domains. Similarly, native NOD2 only interacted with either wild-type or mutant RICK proteins containing the CARD domain. In addition, NOD2 did not associate with other CARD-containing proteins including Apaf-1, caspase-1 and procaspase-9, indicating that the NOD2-RICK interaction is specific and mediated by a homophilic CARD-CARD interaction (Ogura 2001a).

Ogura and colleagues (2001a) determined the essential motifs from the NOD2 molecule required for NF- κ B activation by expressing variant NOD2 proteins, genetically engineered

so that they lacked one or more of the major structural motifs, in HEK293T cells. These investigations showed that both CARD domains were essential for NF- κ B activation, as variant proteins containing only one CARD domain, NOD or LRRs motifs could not induce cellular activation. In addition, these studies also suggested that the LRR motif may play a regulatory role, as engineered NOD2 proteins lacking the LRR motif caused more profound activation of NF- κ B than wild type protein (Ogura 2001, Bonen 2003a).

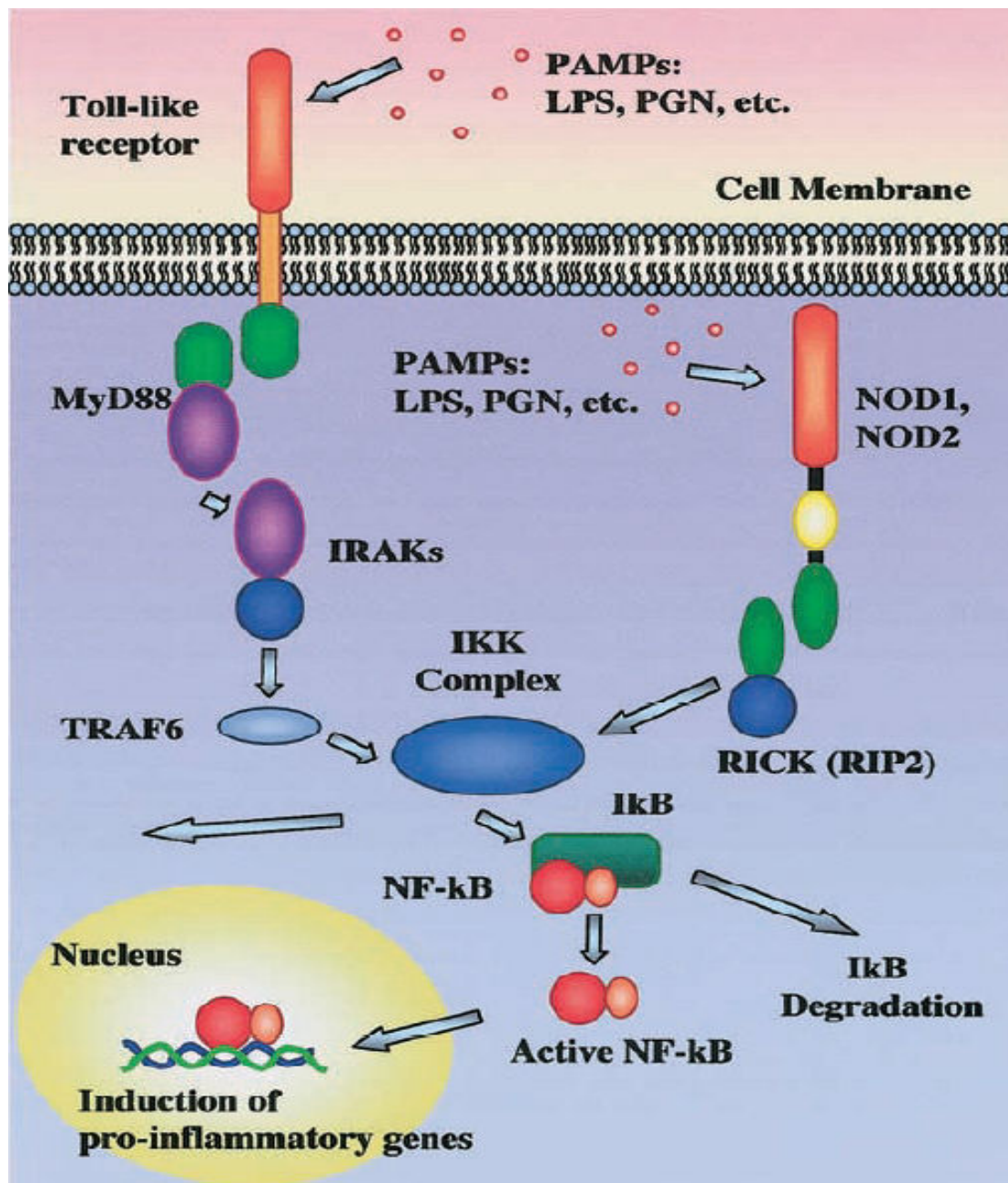


Figure 1.6 Extracellular and intracellular signalling pathways of microbial host interactions. Toll like receptors recognise pathogen associated molecular patterns (PAMPs) and once activated recruit the adapter protein MyD88 which is associated with the interleukin-1

receptor-associated kinase (IRAK). IRAK is then phosphorylated and associates with TNF-receptor-associated factor 6 (TRAF6) which leads to the phosphorylation of I κ B kinase (IKK) and subsequent dissociation of active NF κ B and I κ B. The active NF κ B moves into the nucleus and induces the transcription of various pro inflammatory genes. NOD1 and NOD2 acts intracellularly as receptors for PAMPS and through CARD-CARD interaction, associates with RICK to induce the NF κ B signalling cascade. *Figure adapted from Bonen & Cho 2003*

1.5.3.3.2 NOD2 interacts with the TAK1 signalling pathway

There is emerging evidence that NOD2 activation of NF- κ B is also dependant on transforming growth factor- β -activated kinase 1 (TAK1) (Chen 2004). Transforming growth factor (TGF)- β -activated kinase 1 (TAK1), a MAP kinase kinase enzyme is an essential component of the signalling pathways of many inflammatory cytokines. TAK1 is required for interleukin (IL)-1, tumour necrosis factor- α , IL-18 and TGF- β - mediated NF- κ B activation. (Wang 2001) Pro inflammatory mediators such as IL1 activate TAK1. Activated TAK1 phosphorylates I κ B-related kinases which leads to activation of the NF κ B pathway.

In 2004 Chen and colleagues demonstrated that TAK1 interacts with NOD2 and is required for NOD2 mediated NF- κ B activation. Chen further established using transfected HEK293T cells that the dominant negative form of TAK1 abolished muramyl dipeptide induced NF- κ B activation in NOD2 expressing cells. They then went on to demonstrate that in RICK-deficient embryonic fibroblasts, NOD2 inhibited TAK1-induced NF- κ B activation, suggesting that NOD2 acts in a reciprocal manner, negatively regulating TAK1-induced NF- κ B activation. They further investigated the interaction between NOD2 and TAK1 and suggested that NOD2 interacts with TAK1 through its LRR domain to mediate its inhibitory effect on TAK1 induced NF- κ B activation. Furthermore the wild-type NOD2-LRR more effectively inhibited TAK1-induced NF- κ B activation, than the mutant NOD2 protein encoded by the 3020insC mutation. (Chen 2004).

There is also evidence that TAK1 plays a key role in innate defence. In 2001 Vidal (Vidal 2001) isolated drosophila with null mutations in the *Drosophila dTAK1* gene that encoded *dTAK1* a homolog of TAK1. These flies were viable and fertile but did not produce

antibacterial peptides and were thus highly susceptible to gram-negative bacterial infections. These results suggest that TAK1 may mediate interactions between bacterial products, inflammatory cytokines and NOD2 thereby offering a mechanistic hypothesis of disease pathogenesis in NOD2-affected Crohn's disease.

1.5.3.3.3 Role in apoptosis

Beutler (2001) has suggested that NOD2 may play a role in Crohn's disease-pathogenesis through altered regulated of apoptotic pathways. There is, however, no evidence to suggest that over expression of NOD2 induces apoptosis in cell transfectants, although NOD2 enhances apoptosis induced by caspase-9 expression in these cells (Ogura 2001a). NOD2 and NOD1 promote activation of caspases, however, and their role in apoptosis needs to be evaluated under more physiological conditions including knock-out mice. Indeed, Maeda and colleagues (2005) have shown that, in colitis, an increase in apoptotic lamina propria macrophages is observed in NOD2^{2939iC} mice, which encode for a truncated protein homologous to the Leu1007fsinsC protein. Thus, there remains a possibility that NOD2-dependent apoptosis plays a role in the development of intestinal inflammation, but this remains to be confirmed.

1.6 Paneth cells

1.6.1 Paneth cell Structure

Paneth cells, initially identified by Joseph Paneth in Vienna in 1888 are specialised epithelial cells located at the bases of the crypts of Lieberkühn in the small intestine. (Paneth 1888) There are approximately 20 cells per crypt and their exact function remains unknown. Paneth cells are essential components of the innate intestinal defences and have been proposed to play a regulatory role in intestinal inflammation (Porter 2002, Bevins 2004). Paneth cells are distributed throughout the length of the small intestine but are most numerous in the terminal ileum (Porter 2002). They are not present in the normal colon. They are located adjacent to the stem cell zone and are characterised by the presence of prominent apical secretory granules. They secrete a variety of antimicrobial peptides after exposure to bacteria or bacterial products such as LPS or LTA. These include α -defensins

lysozyme, and secretory phospholipase A₂ (sPLA₂), and result in increased concentrations of antimicrobial products in the intestinal lumen. (Ayabe 2000) These antimicrobial peptides, when released into the gut lumen, are thought to provide protection to the stem cells that are found above Paneth cells in the intestinal crypt. These Paneth cell antimicrobials may play a role in the innate immunity of the gastro-intestinal tract (Porter, 2002) by regulating the composition and number of the intestinal microflora, thus creating unfavourable conditions for the growth of pathogenic bacteria.

There is a link between intestinal inflammation and Paneth cell number. In mouse models of enteric infection, Paneth cell hyperplasia occurs. Furthermore in mouse models of ileocolitis there is Paneth cell metaplasia, and in inflammatory bowel disease, Paneth cell metaplasia is seen in the colon. More recently in the context of intestinal disease, the finding of the NOD2 protein in Paneth cells (Lala 2003, Ogura 2003) has refocused attention on Paneth cells and its possible link to the pathogenesis of Crohn's disease

1.6.2 Paneth Cell function - antimicrobial peptides

1.6.2.1 Alpha defensin expression

Defensins are small (29-45 amino acids in length) cationic peptides that are divided into 2 main families, the α - and β - defensins on the basis of the disulphide bond pairing pattern. They are synthesised as precursor polypeptides that are post translationally processed into mature active peptides. In man the four neutrophil defensins (HNP1-4) were identified first, followed by 2 enteric α -defensins 5 (HD5) and 6 (HD6) which are Paneth cell specific (Elphick & Mahida 2005). HD5 and HD6 are potent antimicrobials peptides which are likely to confer resistance to infection by intestinal pathogens. They are the most abundant antimicrobial peptides found in Paneth cells (Bevins 2004). The α -defensins have a broad spectrum of antimicrobial activity against Gram positive and Gram negative bacteria, fungi and some enveloped viruses. They function by the formation of a membrane spanning pore that eventually leads to lysis of the cell. (Elphick & Mahida 2005) In normal and Crohn's disease-affected terminal ileum, expression of HD5 and HD6 is restricted to Paneth cells. (Cunliffe 2001) The expression of both HD-5 and HD-6 is increased in the colonic mucosa

of inflammatory bowel disease patients through the presence of metaplastic Paneth cells. (Lawrance 2001, Fahlgreen 2003)

Mice cryptdin (defensins) concentrations in intestinal crypts are thought to reach over 1000 times their minimal bactericidal concentrations and thus are thought to create a sterile micro-environment to protect the stem cell compartment and also reduce bacterial colonisation of the mucosa (Elphick & Mahida 2005)

1.6.2.2 Secretory phospholipase A₂ Group IIA (sPLA₂) expression

Secretory phospholipase A₂ Group IIA (sPLA₂) is another antimicrobial enzyme that is a component of Paneth cell granules (Nevalainen 1997). sPLA₂ has been shown to be released into the intestinal lumen after stimulation with bacterial products, including LPS (Harwig 1995). Furthermore purified murine sPLA₂ has been shown to have bactericidal activity against *Salmonella typhimurium* and *Listeria monocytogenes* (Qu 1996). There is increased sPLA₂ expression in inflammatory bowel disease. (Lawrance 2001) It's mode of action is to bind and cleave surface phospholipids.

1.6.2.3 Lysozyme expression

Alexander Fleming first described lysozyme (LZM) in 1922. Lysozyme is an antibacterial enzyme that is found at significant concentrations in many human secretions including tears, breast milk, saliva and gastric and small intestinal secretions. (Elphick and Mahida, 2005) It is expressed in the intestinal tract by gastric and pyloric glands, duodenal Brunner's glands, mononuclear cells and granulocytes but most strongly by Paneth cells. (Stamp 1992) Lysozyme is not expressed in the normal colon but in Ulcerative colitis there is colonic lysozyme expression presumed to be secondary to metaplastic Paneth Cells. (Lawrance 2001) There is no significant difference in Lysozyme expression in small intestinal cells from patients with or without Crohn's disease (Fahlgren 2003). Lysozyme is an enzyme which cleaves the glycosidic bonds that connects N-acetyl muramic acid to N-acetylglucosamine and which stabilises Peptidoglycan thereby resulting in bacterial lysis. LZM is predominantly active against Gram positive bacteria as Gram negative bacteria have an outer membrane which makes them relatively resistant to LZM.

Despite its widespread expression its exact function remains unknown. Its importance in innate immunity however is suggested by the finding of reduced Lysozyme staining Paneth Cells in newborn infants with Necrotising Enterocolitis. (Coutinho 1998) Those authors suggest a deficiency of lysozyme, may render the intestine more susceptible to bacterial infection and bacterial translocation across the mucosa.

1.6.2.4 Tumour necrosis factor alpha (TNF α) expression

TNF α is a potent pro-inflammatory cytokine that is postulated to play a key role in the pathogenesis of Crohn's disease (Beutler 1999). TNF α is an important proinflammatory cytokine that is found in increased concentrations in the mucosa of patients with Crohn's disease (Reinecker 1993) and anti-TNF α immunotherapy is used currently as a highly successful treatment for Crohn's Disease (Targan 1997). The role of Paneth cells is therefore made more interesting as these cells were found to express mRNA to the pro-inflammatory cytokine, TNF- α , in both humans and mice (Keshav 1990)

1.6.3 Paneth Cells and Crohn's disease

Overall, these results suggest that the expression of Paneth cell products may be regulated in inflammatory disease, although the functional consequences of this are unknown. In Crohn's disease, Paneth cells numbers are increased and metaplastic Paneth cells are a feature of Crohn's colitis. There is evidence to suggest that lysozyme and TNF α expression is regulated in IBD, although it is unclear if expression of these products is specifically regulated in Paneth cells.

NOD2 is highly expressed in Paneth cells and NOD2 mutations are associated with terminal ileal disease. Studies in humans and mice have shown that normal NOD2 function is required for optimal defensin expression and therefore impaired defensin expression and regulation may be key features of Crohn's disease pathophysiology. (See later 1.7.1) However the degree to which altered defensin regulation alone can account for the development of Crohn's disease is as yet unclear as spontaneous intestinal disease does not develop in the matrilysin knockout mice (Matrilysin is a protease which cleaves and thus

activates α defensins in mice) Furthermore Mori-Akiyama (2007) and colleagues recently developed the SOX 9 knock out mice which have no functioning Paneth cells and these mice were also outwardly normal, of the same weight as healthy controls and fertile. They also did not develop spontaneous intestinal disease.

1.7 Effect of Crohn's Disease associated NOD2 mutations

There is considerable current interest into the mechanism whereby an abnormal NOD2 protein leads to Crohn's disease. Possible mechanisms involve a defective recognition of bacterial products by NOD2 in Paneth cells leading to a dysregulated Paneth cell mediated bacterial response and abnormal microbial colonisation of the small bowel which may provoke chronic inflammation via adaptive immune mechanisms.

1.7.1 Effect of Crohn's Disease associated NOD2 mutations on Paneth Cell Function

The functional importance of defensins has been illustrated recently in a number of experiments in both mouse and human models. NOD2 deficient mice generated in two independent laboratory are outwardly healthy and do not develop histological evidence of inflammation after 6 months observation (Kobayashi 2005, Pauleau 2003). However when challenged with intragastric *Listeria monocytogenes* the NOD^{-/-} mice had greater susceptibility to infection as shown by significantly greater numbers of bacteria recovered from the liver and spleen compared to wild type controls. Interestingly when challenged with *Listeria monocytogenes* intraperitoneally or intravenously there was no difference between the NOD2 ^{-/-} mice and wild type controls. Furthermore NOD2 ^{-/-} mice were found to have reduced Paneth cell expression of α -defensins cryptidin 4 and cryptidin 10 and this was further reduced after intragastric infection with *Listeria monocytogenes* (Kobayashi 2005). This suggests that a functioning NOD2 plays a key role in mediating protection of the intestinal mucosa against bacterial infection possibly by mediating optimal defensin response.

In a further experiment using transgenic mice the functional importance of α - defensins was again demonstrated. In transgenic mice which expressed human HD-5 in intestinal Paneth cells Salzman and colleagues demonstrated increased protection against *Salmonella*

typhimurium. (Salzmann 2003) After lethal oral *Salmonella typhimurium* challenge there was a reduction in the bacterial burden in the intestinal lumen and faeces, reduced bacterial translocation to the spleen and higher survival rates in the HD5 transgenic mice. Interestingly however HD5 transgenic mice did not have a survival advantage when injected intraperitoneally with *Salmonella typhimurium*, indicating that the effect of HD5 is again mediated in the intestinal lumen. Furthermore using a matrilysin knockout mice (matrilysin – a protease which cleaves and thus activates α defensins in mice) Wilson and colleagues demonstrated a defect in the microbicidal activity in vivo after oral inoculation with *Escherichia Coli* as evidenced by increased numbers of bacteria in the distal small intestine. In addition these knock out mice were more susceptible to invasive pathogens such as *Salmonella typhimurium*, in comparison to wild type mice. (Wilson 1999)

The majority of human data comes from Wekhamp and colleagues who originally demonstrated that defensins are differentially expressed in Crohn's disease and ulcerative colitis. (Wekhamp 2004) Following the identification that Paneth cells express NOD2, (Lala 2003) Wekhamp hypothesised that NOD2 may regulate or influence Paneth cell number or Paneth cell defensin expression. They examined 45 Crohn's disease patients (24 with NOD2 variants and 21 without) and 12 controls and showed that ileal expression of HD-5 and HD-6, but not sPLA₂ or lysozyme were diminished in Crohn's affected ileum, and the decrease was significantly more pronounced in patients with NOD2 mutations. In the colon, HD-5, HD-6, and sPLA₂ were increased during inflammation in wild-type but not in NOD2 mutated patients. In both the colon and ileum, proinflammatory cytokines and lysozyme were unaffected by NOD2 status (Wekhamp 2004)

Wekhamp concludes that as alpha defensins are important in the mucosal antibacterial barrier, their diminished expression may explain, in part, the bacterial induced mucosal inflammation and ileal involvement of Crohn's disease, and that this may be mediated by inherited mutations in NOD2.

1.7.2 Effect of Crohn's Disease associated NOD2 mutations on MDP mediated cell responses

1.7.2.1 Transfection Studies

Bonen and colleagues (2003a) showed that PGN-induced cellular activation is diminished in mammalian cells expressing mutant NOD2 proteins. Using transfected HEK293T cells they showed that NF- κ B activation was reduced or absent in PGN-treated cells that expressed mutant NOD2 proteins. In a similar way, Girardin and colleagues (Girardin 2003) showed that mutant NOD2 proteins do not mediate NF- κ B activation in response to PGN and MDP stimulation. Both groups showed that no MDP-induced NF- κ B activation was noted in HEK293T cells expressing the Leu1007fsinsC-encoded NOD2 mutant protein whereas lesser degrees of activation were noted in cells expressing the Arg702Trp- and Gly908Arg-encoded NOD2 proteins.

Inohara and colleagues (2003) verified this finding in mammalian cells. These investigators obtained primary peripheral blood mononuclear cells (PBMC) from healthy volunteers and Crohn's disease patients that were either homozygous or heterozygous for the Leu1007fsinsC mutation. Using electrophoretic mobility shift assay and quantitative real-time PCR, they showed that MDP did not induce NF- κ B activation in PBMC which were homozygous for the frameshift NOD2 mutation. MDP did however induce NF- κ B activation in PBMC from normal controls or patients who were heterozygous for the NOD2 mutations. This was in contrast to LPS stimulation which induced NF- κ B activation in all PBMC regardless of NOD2 status, further demonstrating that NOD2 mediates the host cellular response to MDP.

Interestingly all three of the major risk alleles have comparable allele frequencies and genetic risk for heterozygotes and homozygotes, but only the Leu1007fsinsC has been shown to have complete loss of function in transfection studies (Inohara 2003, Bonen 2003a)

1.7.2.2 Murine Studies

The role of MDP in the pathogenesis of NOD2-associated Crohn's disease has been examined using mouse studies by two groups - Murray and Maeda (Pauleau 2003, Maeda 2005).

Murray in his NOD2^{-/-} mice showed reduced secretion of proinflammatory cytokines IL-6, IL-12 and TNF α in Bone marrow derived monocytes stimulated with MDP from ^{-/-} mice compared to controls. (Pauleau 2003) The response to other TLR stimulation was comparable in both ^{-/-} and control mice.

Maeda generated mice whose NOD2 encoded for a truncated NOD2 protein lacking the last 33 amino acids, which is homolog of the human NOD2 protein that is encoded by the frameshift mutation. In this model, in contrast to previous studies MDP stimulation caused increased levels of NF- κ B activation in macrophages isolated from NOD2^{2939iC} mice relative to macrophages from wild-type mice. Furthermore, the expression of several NF- κ B target genes was increased in MDP-treated NOD2^{2939iC} macrophages relative to wild-type controls and MDP also induced increased secretion of IL-1 β from NOD2^{2939iC} macrophages relative to wild-type controls. (Maeda 2005)

1.7.2.3 Human Studies

Li (Li 2004) first examined the effects of MDP mediated activation on primary peripheral mononuclear cells stratified on NOD2 genotype. They demonstrated that by microarray, MDP induced a broad array of transcripts, including IL-1 β and IL-8. Furthermore they demonstrated a reduced transcriptional response to MDP in mononuclear cells homozygous for the frameshift mutation. At low MDP doses (10ng/ml) they observed no induction of IL-8 protein in all homozygotes but using higher doses of MDP (1 μ g/ml) only those mononuclear cells homozygous for the frameshift mutation showed no induction of IL-8 protein. In the same paper Li also went on to demonstrate that in wild type mononuclear cells that MDP plus TNF α together causes a synergistic induction of IL-1 β secretion and

that this is absent in Leu1007fsinsC homozygotes despite marked induction of IL-1 β mRNA. They suggest that this shows a post –transcriptional dependency on the NOD2 pathway for IL-1 β secretion.

Netea (Netea 2004) alternatively demonstrated a defective release of IL-10 from blood mononuclear cells homozygous for the frameshift mutation, after stimulation with TLR2 ligands (PGN and Pam3Cys-KKKK) but not with TLR4 ligands (LPS). They also demonstrated decreased anti – inflammatory cytokine release after stimulation with *Bacteroides* species and concluded that the resulting pro- inflammatory cytokine bias could be responsible for the inflammation seen in Crohn’s disease.

Van Heel (van Heel 2005) also analysed the cytokine response of peripheral blood mononuclear cells to MDP. They demonstrated that MDP alone induced little TNF α or IL - 1 β but strong IL-8 secretion. Furthermore they went on to demonstrate that MDP substantially upregulated the secretion of TNF α or IL -1 β induced by Toll like receptors 2, 4, 5 and 6. These effects were not seen in mononuclear cells homozygous for the common NOD2 mutations. These results will be discussed more fully in Chapter 4.5

1.8 Aims

The most intriguing question therefore remains concerning the mechanism whereby mutations in the *NOD2* gene predispose towards Crohn’s disease. Thus far functional studies of the NOD2 protein have relied heavily on the use of transfected cell lines and murine models. Most of these studies have shown that mutant NOD2 proteins, especially those encoded by the frameshift mutation, mediate a decreased NF- κ B response. Yet Crohn’s disease is characterised by increased NF- κ B activation with downstream effects on inflammatory cytokine production. At the time of doing my research the functional effect of the established NOD2 risk alleles had not yet been examined in primary human mononuclear cells. The aim of my research was therefore to study the effect of inherited mutations in the *NOD2* gene on cellular responses to bacterial stimuli using freshly extracted primary human monocytes both from healthy controls and from patients with Crohn’s disease carrying the common *NOD2* gene mutations.

With the more recent discovery showing NOD2 expression in Paneth cells and with emerging evidence that Paneth cell antimicrobial products may be regulated in NOD2 associated Crohn's disease, I investigated whether another Paneth cell derived antimicrobial enzyme, lysozyme, plays a role in inducing cellular activation in intestinal epithelial cells. Most functional studies of NOD2 function have relied on synthetic MDP and PGN but in biological systems, muropeptides (of which MDP is a commercially available example) are generated by the enzymatic action of lysozyme on the PGN component of the bacterial cell wall. I therefore wanted to investigate the effect of lysozyme and lysozyme generated muropeptides from various bacterial species on cellular inflammatory responses by measuring the transcriptional activity of specific inflammatory cytokines in intestinal epithelial cells.

The original hypotheses were that carriage of a mutant NOD2 gene would affect the response of PBMC and Paneth cells to bacterial stimulation.

The specific objectives of this study are:

1. To determine the effect of various bacterial antigens on inflammatory cytokine expression in PBMC freshly extracted from healthy volunteers and Crohn's disease affected patients carrying wild-type and mutant *NOD2* genotypes.
2. To examine the effect of MDP pre treatment 'priming' on inflammatory responses in PBMC extracted from healthy volunteers and Crohn's disease affected patients carrying wild-type and mutant *NOD2* genotypes.
3. To examine the effect of different *mycobacteria* stimulation on PBMC extracted from healthy volunteers and Crohn's disease affected patients carrying wild-type and mutant *NOD2* genotypes.
4. To examine the effect of lysozyme generated muropeptides on inflammatory responses in intestinal epithelial cells by measuring the transcriptional activity of specific inflammatory cytokines.
5. To examine whether lysozyme generated muropeptides from different bacterial species provoked differing effects on inflammatory signalling in intestinal epithelial cells.

Chapter Two

2. Materials and Methods

2.1 Materials

A list of reagents and suppliers is attached - Appendix C

2.2 Molecular biology Techniques

2.2.1 Isolation of peripheral blood mononuclear cells (PBMC)

Reagents:

1. Histopaque® 1077: A solution containing polysucrose and sodium diatrizoate, adjusted to a density of 1.077 g/mL.
2. Hanks' balanced salt solutions (HBSS)
3. 0.5M EDTA
4. Macrophage serum free media

Peripheral blood mononuclear cells (PBMC) were harvested, using Ficoll density gradient centrifugation.

Method:

20mls of blood was collected from human healthy volunteers or from patients attending the Inflammatory Bowel Disease clinic at the Royal Free Hospital, London. The project was approved by the joint Ethics Committee of the Royal Free Hampstead NHS Trust and Royal Free and University College Medical School (London, UK). The fresh collected blood was put into anti-coagulant tubes containing K₃EDTA (BD Diagnostics) for immediate transfer to the laboratory. Once in the laboratory the whole blood was transferred to a 50 ml-centrifuge tube and 10ml Hanks' balanced salt solutions (HBSS) (with 2.5mM EDTA to aid cell separation) was added to make a total solution of 30mls. This was then very carefully layered onto 15mL Histopaque® 1077 which was in a separate 50 mL-centrifuge tube, and then centrifuged at 1400 rpm (409 g) for 20 minutes at 20°C with the brake off. Following centrifugation, mononuclear cells form a distinct layer at the plasma- Histopaque® interface, and red blood cells and neutrophils form a pellet at the

base of the tube. The layer of mononuclear cells was then carefully aspirated and placed in fresh 50 mL-centrifuge tubes. HBSS was added to make the final volume 50 mL, and the samples were centrifuged at 1400 rpm (409g) for 10 minutes at 4°C with the brakes on. After discarding the supernatant containing contaminating platelets, the PBMC pellet was re-suspended in 50 mL HBSS, and centrifuged at 1200 rpm (301 g) for a further 7 minutes at 4°C with the brakes on. This wash in 50mL HBSS was repeated twice more, to remove contaminating platelets. A final wash was then performed in 5mls Macrophage serum free media and following that final wash, the PBMC were suspended in 3mls Macrophage serum free media and counted using an improved Neubauer counting chamber. The monocytes were then suspended, at a concentration of 2×10^6 cells/ml, and 2mls put into each well of a 6 well plate and incubated at 37°C in a 5% CO₂, 95% air atmosphere. This gave a total of 4×10^6 cells well.

2.2.2 Bacterial stimulation of PBMC

Reagents

1. Muramyl diPeptide (MDP) N-acetylmuramyl-L-alanyl-D-isoglutamine
2. Peptidoglycan (PGN) *micrococcus luteus* Sigma-Aldrich – category number 53243, *staphylococcus aureus* Sigma-Aldrich – category number 77140
3. Lipopolysaccharide (LPS)
4. Sonicated *Mycobacteria vaccae* (Strain NCTC11659 and *Mycobacteria tuberculosis* (Strain H37RV) gift of Dr Sanjay Lala

After being incubated at 37°C in a 5% CO₂, 95% air atmosphere for one hour the 4×10^6 PBMC in each well were stimulated with the appropriate bacterial stimuli. The bacterial stimuli was diluted into the correct concentration in warmed Macrophage serum free media before being added to the PBMC at time zero. Each well finally contained 4×10^6 cells in 3 ml of Macrophage serum free media. The 6 well plates containing the PBMC and bacterial stimuli were then incubated at 37°C in a 5% CO₂, 95% air atmosphere for a further 1,2,4 or 6 hours depending on the experiment. At harvesting, the PBMC were recovered by aspiration and mechanical scraping to ensure that both adherent and non-adherent cells were obtained. For RNA extraction, the recovered PBMC were lysed in Trizol® reagent.

2.2.3 RNA extraction

Reagents:

1. Trizol® reagent
2. Isopropanol
3. 70% ethanol
4. Nuclease-free water/ DEP-C Water
5. Chloroform

Method:

RNA extraction from intestinal epithelial cells and peripheral blood mononuclear cells (PBMC) was performed using Trizol® reagent. For RNA extraction from PBMC cultured in 6-well plates, the culture medium containing non-adherent lymphocytes was transferred to clean microfuge tubes and centrifuged at 13 000 rpm (16 100 g) for 5 minutes at 4°C. This supernatant was then stored at -20°C for future protein analysis. 600 µL Trizol reagent was added to each well and after agitation the solution transferred to a clean microfuge tube. The remaining adherent mononuclear cells in the 6-well culture plates were lysed using a further 400 µL Trizol, and this solution was added to the microfuge tubes containing the lysed lymphocytes so that the entire cell lysate was present in 1 mL Trizol reagent.

For intestinal epithelial cells the supernatant was discarded and 500µL of Trizol was added and after agitation the solution transferred to a fresh microfuge tube. A further 500µL of Trizol was then added to ensure collection of all cells and the final solution was again 1ml.

Following that 400 µL chloroform was added to each microfuge tube, and mixed for 15 seconds using a vortex mixer till the samples were thoroughly mixed. The samples were then centrifuged at 14 000 rpm (20 817 g) for 10 minutes at 4 °C to allow for phase separation. The upper or RNA-containing aqueous phase was removed by careful aspiration, taking care to avoid the protein interface, and transferred to fresh tubes. An equal volume of isopropanol was then added to each tube. After mixing the contents, the sample was incubated at -20°C for 30 minutes to allow for RNA precipitation. Next, the tubes were centrifuged at 14 000 rpm (20 817 g) for 15 minutes at 4 °C to collect the RNA pellet. The supernatant was then discarded and the RNA pellet was washed in 500 µL 70%

ethanol and the tubes were centrifuged again at 14 000 rpm (20 817 g) for 10 minutes at 4°C. After discarding the supernatant, the RNA pellet was air dried and resuspended in 22 µL nuclease-free water. 20 µL of RNA solution was used for cDNA synthesis, and 2 µL of solution used to quantify and assess RNA integrity by spectrophotometry and agarose gel electrophoresis.

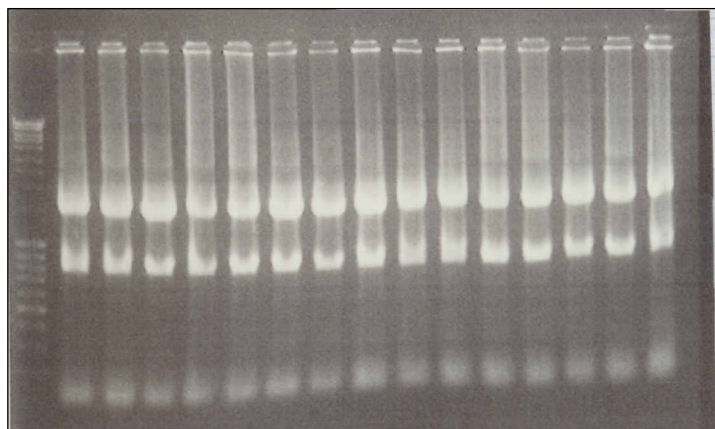


Figure 2.1 – Example of agarose gel electrophoresis on RNA extracted from PBMC

2.2.4 Synthesis of cDNA

Reagents:

1. Oligo(dT)₁₂₋₁₈ primer (0.5 µg/µL; Invitrogen Life Technologies)
2. 10 mM dNTP
3. 5X First Strand Buffer [250 mM Tris-HCl (pH 8.30, 375 mM KCl, 15 mM MgCl₂; Invitrogen Life Technologies]
4. Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT) (200 units/µL; Invitrogen Life Technologies)
5. Nuclease-free water

Method:

For cDNA synthesis, 2 µg of total RNA, in 20 µl of nuclease-free water, and 0.5 µg oligo (dT) primer (Life Technologies Ltd), were heated to 65° C for 5 minutes to dispose the 2° structure and then rapidly cooled on ice. Reverse transcription was performed at 37.0°C for 2hours, in a 50 µl reaction volume, according to the following protocol:

RNA (2 µg)	20 µL
Oligo (dT) primer	1 µL
10 mM dNTP	2.5 µL
5X First Strand buffer	10 µL
MMLV-RT	2 µL
Nuclease-free water	<u>14.5 µL</u>
Reaction volume	50.0 µL

Synthesised cDNA was stored at -20°C

2.2.5 Primer Design

Primers were designed after visually examining the nucleotide sequences that are accessible on PubMed, a service provided by the United States National Library of Medicine (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>). They were designed to be intron spanning to distinguish mRNA from possible contamination from genomic DNA. They were 20 nucleotide bases in length and designed to anneal at 60°C during PCR amplification. All oligonucleotide DNA primers were synthesised and purified by reverse-phase High Pressure Liquid Chromatography (HPLC) by Thermo Electron GmbH, Germany. Primers were synthesised to a scale of 0.02 µmoles, and shipped in a dry or lyophilised form. The oligonucleotide primers were reconstituted in nuclease-free water to 100 µM concentration, and stored at -20 °C. Amplicons were diluted to a final concentration of 10 µM for use in PCR amplification. Table 2.1 lists the oligonucleotide primers used in RT-PCR and quantitative RT-PCR reactions.

2.2.6 Quantitative Real Time Polymerase Chain Reaction (q-PCR)

Reagents:

1. 10 µM sense and anti-sense primers (Primers synthesised by Thermo Electron GmbH, Germany)
2. 25 mM MgCl₂

3. 10 mM deoxynucleotide triphosphate (dNTP) mix (containing 10 mM dATP, 10 mM dTTP, 10 mM dCTP and 10 mM dGTP)
4. 10X Qiagen PCR Buffer (Contains Tris-Cl, KCl, (NH₄)₂SO₄, 15 mM MgCl₂; pH 8.7. Manufacturer does not disclose information regarding concentrations)
5. Q-Solution (Qiagen; manufacturer does not disclose details of composition)
6. *Taq* polymerase (5U/ μ L; Qiagen)
7. SYBR green (Biogene)
8. DEPC-treated water

Method

Quantitative real-time PCR was performed using the Rotor-Gene amplification system (Biogene, Kimbolton, UK). RT-PCR was performed, in a 20 μ L reaction volume, according to the following protocol. Each 20 μ L PCR reaction contained 5.25 μ L of RNase free water, 3 μ L of cDNA, 1 μ M of sense and anti-sense primer, 1.5mM MgCl₂, 1 μ L of dNTP's (5mM each of dATP, dTTP, dCTP and dGTP) 1 μ L SYBR green (Biogene; final concentration 1:60 000), 2 μ L 10X Qiagen PCR Buffer (Qiagen, Crawley, UK), 4 μ L of Q-solution (Qiagen) and 0.25U *Taq* polymerase (Qiagen) according to the following protocol.

Protocol

Intron-spanning primer pairs were used to amplify each cDNA

cDNA	3 μ L
10 μ M primers (sense + anti-sense)	2 μ L
25 mM MgCl	1.5 μ L
10 mM d NTP mix	1 μ L
<i>Taq</i> polymerase (5U/ μ L)	0.25 μ L
10x PCR Buffer solution	2 μ L
Q-Solution	4 μ L
SYBR green	1 μ L
DEPC-treated water	<u>5.25</u> μ L
Reaction volume	20.0 μ L

Synthesised cDNA was initially denatured at 94 °C for 3 minutes, and amplified using 40 cycles of denaturation at 94 °C, annealing at 60 °C for 30 seconds, and extension at 72°C for 60 seconds. The data were expressed as the ratio of NOD2 to GAPDH. Specificity of the desired PCR products was determined by melting curve analysis and confirmed by agarose gel electrophoresis and ethidium bromide staining.

2.2.7 Gel electrophoresis

The size of all PCR products was verified by agarose gel electrophoresis and UV transillumination. A 1% agarose gel was prepared by dissolving 0.6g agarose in 60 mL water. This solution was heated, using a microwave oven (for 3 minutes using the low power setting), and then poured into an electrophoresis box that was specifically used for either RNA or DNA analysis respectively. The gel was allowed to set at room temperature and, once polymerised, was covered with nuclease free water. The cDNA samples were loaded and the gel was run for 25minutes at 65 V (5 V/ cm) prior to UV transillumination.

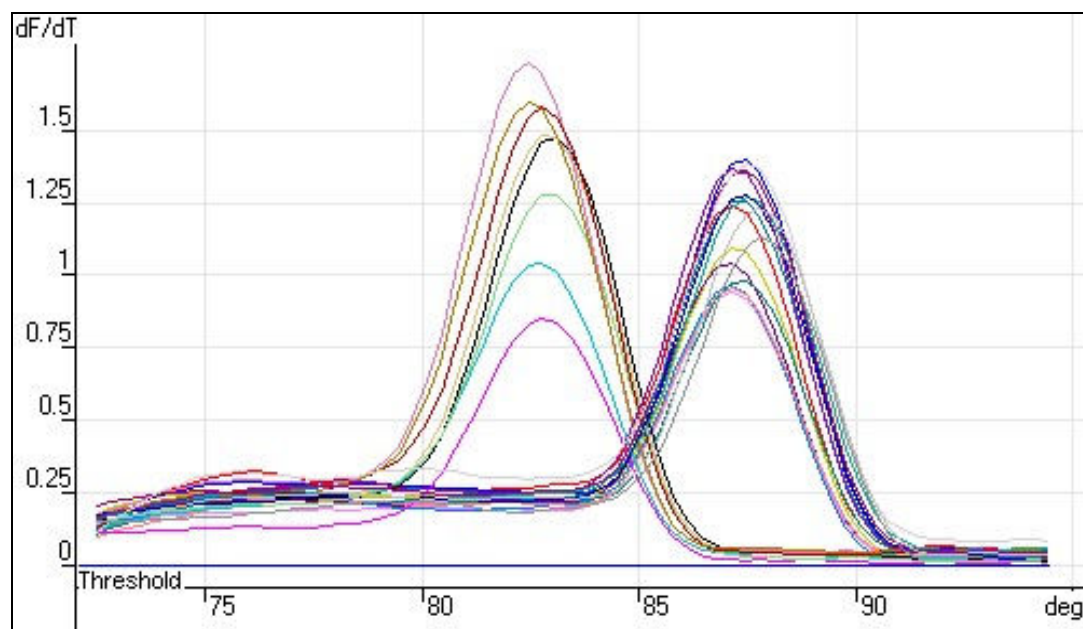


Figure 2.2 – Example of melt curve produced-peripheral blood monocytes stimulated by bacterial ligands: $\text{TNF}\alpha$, $\text{IL1}\beta$ and GAPDH expression

Table 2.1 Oligonucleotide primers

Primer	5' – 3' sequence
IL-8 2878F	CTT TCT GAT GGA AGA GAG CTC
IL-8 3968R	GTC CTC ACA ACA TCA CTG TG
IL1b 5724F	GGT GTT CTC CAT GTC CTT TG
IL1b 6779 R	GGA AGA CAC AAA TTG CAT GG
HD6 1471F	CCA CTC CAA GCT GAG GAT G
HD6 2697R	TAG GAC ACA CGA CAG TTT CC
NOD2 115520F	GCG CGA TAA CAA TAT CTC AGA
NOD2 122313R	CAG AGT TCT TCT AGC ATG ACG
GAPDH 3543F	CGA GAT CCC TCC AAA ATC AAG
GAPDH 4610R	G AGC TTG ACA AAG TGG TCG
Lysozyme 4353F	GCT GCA AGA TAA CAT CGC TG
Lysozyme 5417R	ACC TTT CAC TTA ATT CCT ACT CCC
TGFβ 1740F	ACA TTG ACT TCC GCA AGG AC
TGFβ 2269R	AAG GAA TAG TGC AGA CAG GC
TNFα 1814F	GAG TGA CAA GCC TGT AGC CC
TNFα 2380R	TGG CAG AGA GGA GGT TGA CC

F: forward primer; R: reverse primer. All primers pairs were designed to span introns, and the expected genomic DNA and mRNA sizes (number of nucleotide base pairs) is indicated. All numbers indicate the starting nucleotide position of the primer, listed according to the GenBank accession number.

2.2.8 Cell culture

Reagents:

1. 100x Antibiotic antimycotic solution: 10 000 U penicillin, 10 mg streptomycin, and 25 µg amphotericin B per mL)
2. 10x Trypsin-EDTA solution: 5.0 g porcine trypsin, 2.0 g EDTA-4 Na in 0.9% NaCl; cell culture tested
3. Trypsin solution: 1 mL 10x Trypsin-EDTA solution and 9 ml Dulbecco PBS
4. Dulbecco PBS
5. Dulbecco's modified Eagle medium (DMEM)
6. DMEM/F12 medium
7. 10% foetal calf serum
8. 200 mM L-Glutamine
9. HT-29 intestinal epithelial cells (ATCC Number HTB-38)
10. SW480 intestinal epithelial cells (ATCC Number CCL-228)

HT29 intestinal epithelial cells were obtained from Dr. A Bromfield (Department of Medicine, Royal Free and University College Medical School, London). This investigator had originally purchased the cells from LCG Promochem, the European distribution partner for American Type Culture Collection (ATCC) products. SW480 cells were purchased directly from LCG Promochem.

2.2.8.1 Propagation of intestinal epithelial cell lines

Human colon cancer cell line HT29 (American Type Culture Collection, Manassas, USA) was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum, 2mM L-Glutamine, 1 U/mL penicillin, 1 µg/mL streptomycin and 2.5 ng/mL amphotericin B, at 37°C in a 95% air/ 5% CO₂ atmosphere. SW480 cells were cultured in DMEM/F12 medium supplemented with 10% foetal calf serum, 1 U/mL penicillin, 1 µg/ml streptomycin and 2.5 ng/mL amphotericin B, at 37°C in a 95% air/ 5%

CO₂ atmosphere. All reagents used for cell culture were purchased from Sigma Chemical Company, Poole, UK.

Cell lines were propagated after reaching 80-90% confluence in T75 flasks (usually after one week of culture). The growth medium was poured from the flask, and the cells were twice washed with 10 mL Dulbecco PBS. After pouring out the PBS, 5 mL of trypsin solution was added to the flask. After 1 minute, approximately 4.5 mL of the trypsin solution was removed from the flask, and the flask was incubated at 37.0°C for approximately 10 minutes.

Following trypsinisation, the cells were visualised under a microscope to confirm cell detachment from the plastic walls of the flask. The detached cells were thoroughly resuspended in 8 mL of appropriate fresh culture medium. After calculating the cell count, using an improved Neubauer counting chamber, cells were seeded into fresh T75 culture flasks according to the following concentrations: $2-4 \times 10^4$ cells/ cm² for HT-29 cells, and 10^4 cells/ cm² for SW480 cells. Fresh culture medium was added to the T75 flask so that cells were allowed to propagate in 10 mL growth medium; the growth medium was replenished twice weekly.

All cell lines were checked and found to be free from mycoplasma infection by Ms Demetra Mavri (Research assistant, Centre for Hepatology, Royal Free and University College Medical School, London).

Table 2.3 Seeding concentrations used to propagate intestinal epithelial cells.

Cell type	Seeding concentration (cells/ cm ²)	Cell yield from trypsin- treated confluent cells cultured in T75 flasks
HT-29	$2-4 \times 10^4$	$2-3 \times 10^7$
SW480	1×10^4	$2-3 \times 10^7$

2.2.8.2 Bacterial stimulation of intestinal epithelial cell lines

Reagents

- 1 Muramyl diPeptide (MDP) N-acetylmuramyl-L-alanyl-D-isoglutamine
- 2 Peptidoglycan (PGN) *micrococcus luteus* Sigma-Aldrich – category number 53243, *staphylococcus aureus* Sigma-Aldrich – category number 77140, *bacillus subtilis* Sigma-Aldrich – category number 69554
- 3 Lipopolysaccharide (LPS)
- 4 Lysozyme (LZM)
- 5 E.Coli LF82

Cells were grown to 90% confluence and as before the growth medium was poured from the flask, and the cells were twice washed with 10mL Dulbecco PBS. 5 mL of trypsin solution was then added to the flask for 1 minute and then approximately 4.5 mL of the trypsin solution was removed from the flask. The flask was then incubated at 37.0°C for approximately 10 minutes. Following trypsinisation, the cells were visualised under a microscope to confirm cell detachment from the plastic walls of the flask. The detached cells were then thoroughly resuspended in 12 ml of fresh culture medium and plated 1 ml each into 2 x 6 well plates each well already containing 2ml of warmed fresh culture medium. They were incubated at 37°C in a 95% air/ 5% CO₂ atmosphere for 24 hours. Once they had reached approximately 70% confluence in each well the medium was removed, cells were washed with PBS and then stimulated with the correct concentration of bacterial stimulation and/or freshly diluted LZM in warmed fresh medium to make a total of 3 ml in each cell. LZM was freshly made for each experiment. The dose chosen was initially 100 times physiological levels – 64mg/ ml and then increased to 100mg/ml further for ease of experimental technique. They were then incubated for a further set time period before the RNA was extracted using Trizol reagent.

2.3 Clinical Data Collection

I met all patients involved in this study personally through the Inflammatory Bowel Disease Clinic at The Royal Free Hospital London. They were interviewed at time of venesection and I later reviewed each set of notes and recent haematology and biochemistry results to ensure correct clinical information was obtained. The project was previously approved by the joint Ethics Committee of the Royal Free Hampstead NHS Trust and Royal Free and University College Medical School (London, UK).

2.3.1 NOD2 Genotyping

Genomic DNA had previously been isolated from venous blood of 150 patients attending the Inflammatory Bowel Disease Clinic at the Royal Free Hospital, London. Using an allele-specific polymerase chain reaction, followed by agarose gel electrophoresis Dr Manisha Abeya, (MD student, Department of Medicine, Royal Free and University College Medical School, London) had determined the NOD2 status of each of these patients. All NOD2 mutant samples were confirmed by DNA sequencing.

2.4 Statistical analysis

Values in the text and figures are expressed as the mean \pm S.E.M. (Standard error of the mean). Statistical differences between means were determined using Student's t-test and considered significant if $p < 0.05$. All analyses were performed using GraphPad Prism version 3.02 (25 April 2000) (GraphPad, San Diego, CA)

Chapter Three

3.0 Determining the effect of direct bacterial stimulation on peripheral blood mononuclear cells (PBMC) cytokine expression

3.1 Introduction

Crohn's Disease is an inflammatory condition which can affect the entire gastrointestinal tract and whose aetiology remains unclear but is thought to be due to an excessive response to a component of the bacterial flora in a genetically susceptible host. (Bouma 2003) Mutations in the NOD2 gene are strongly associated with Crohn's disease. NOD2 is expressed intracellularly in monocytic cells and in Paneth cells of the small intestine. NOD2 has been shown to recognize MDP a component of bacterial cell wall peptidoglycan that is present in most bacteria. The challenge has been to understand how mutations in NOD2 gene are involved in the pathogenesis of Crohn's disease. I therefore investigated peripheral blood mononuclear cells (PBMC) response to bacterial stimuli and the effect of inherited mutations in the NOD2 gene on that response.

3.1.1 Bacterial Stimulation of PBMC from Healthy Controls – Initial Cytokine Studies

Initial studies in two healthy individuals involved freshly isolated PBMC being stimulated in serum free media with either Muramyl dipeptide (20µg/ml), PGN *m.luteus* (20µg/ml) or PGN *s.aureus* (20µg/ml) for 2 hours. After extraction of RNA and cDNA synthesis, quantitative real time PCR was performed looking at cytokine expression. Results were then normalised for Glutaryldehyde phosphate dehydrogenase (GAPDH) a house keeping gene. The cytokines we examined included TNF α , IL-1 β and IL-8. In Crohn's disease, mucosal inflammation is mediated by an excessive T helper 1 (T_H1)-cell response that is characterized by the cytokine tumour necrosis factor α (TNF α). Interleukin-1 β (IL-1 β) and interleukin-8 (IL-8) are cytokines involved in the immediate innate immune response. IL-8, a member of the CXC chemokine family, is an important activator and chemo-attractant for

neutrophils and has been implicated in the pathogenesis of a variety of inflammatory diseases, including IBD, asthma and sepsis (Standiford 1990, Yoshimura 1987)

Figure 3.1 shows that MDP induced minimal upregulation 4.2 fold of TNF α cytokine expression whilst PGN from both *m.luteus* and *s.aureus* induced a mean 24 and 22 fold increase in TNF α expression from normal PBMC.

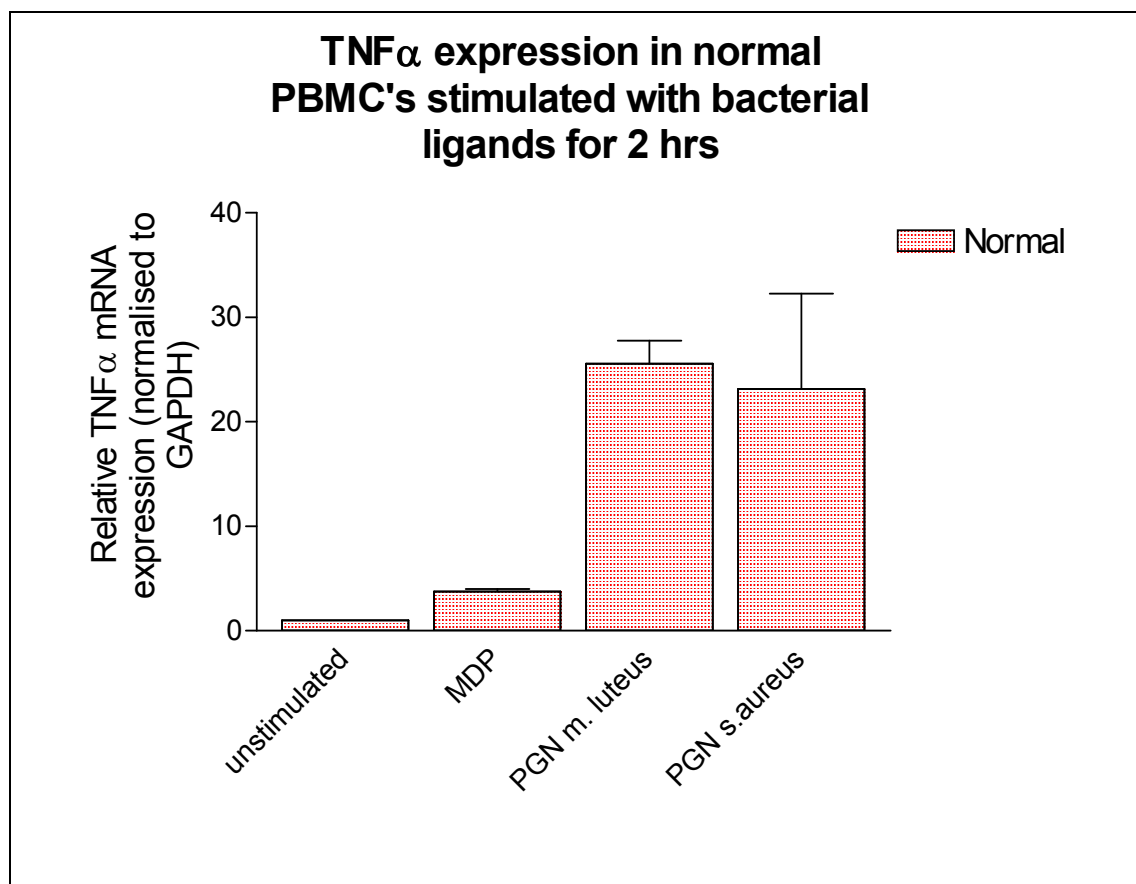


Figure 3.1 – Stimulation of peripheral blood monocytes by bacterial ligands: TNF α expression

I also wanted to determine the effect of bacterial stimulation on the expression of the regulatory cytokine transforming growth factor β (TGF β) as well as NOD2 and lysozyme expression (LZM). TGF β is a cytokine produced by suppressor T cell subsets, which ameliorate T_H1 mediated intestinal inflammation which are important in CD pathogenesis. There was no significant response to bacterial stimuli in terms of transforming growth factor β (TGF β), NOD2 or LZM expression in PBMC from healthy volunteers. Figure 3.2

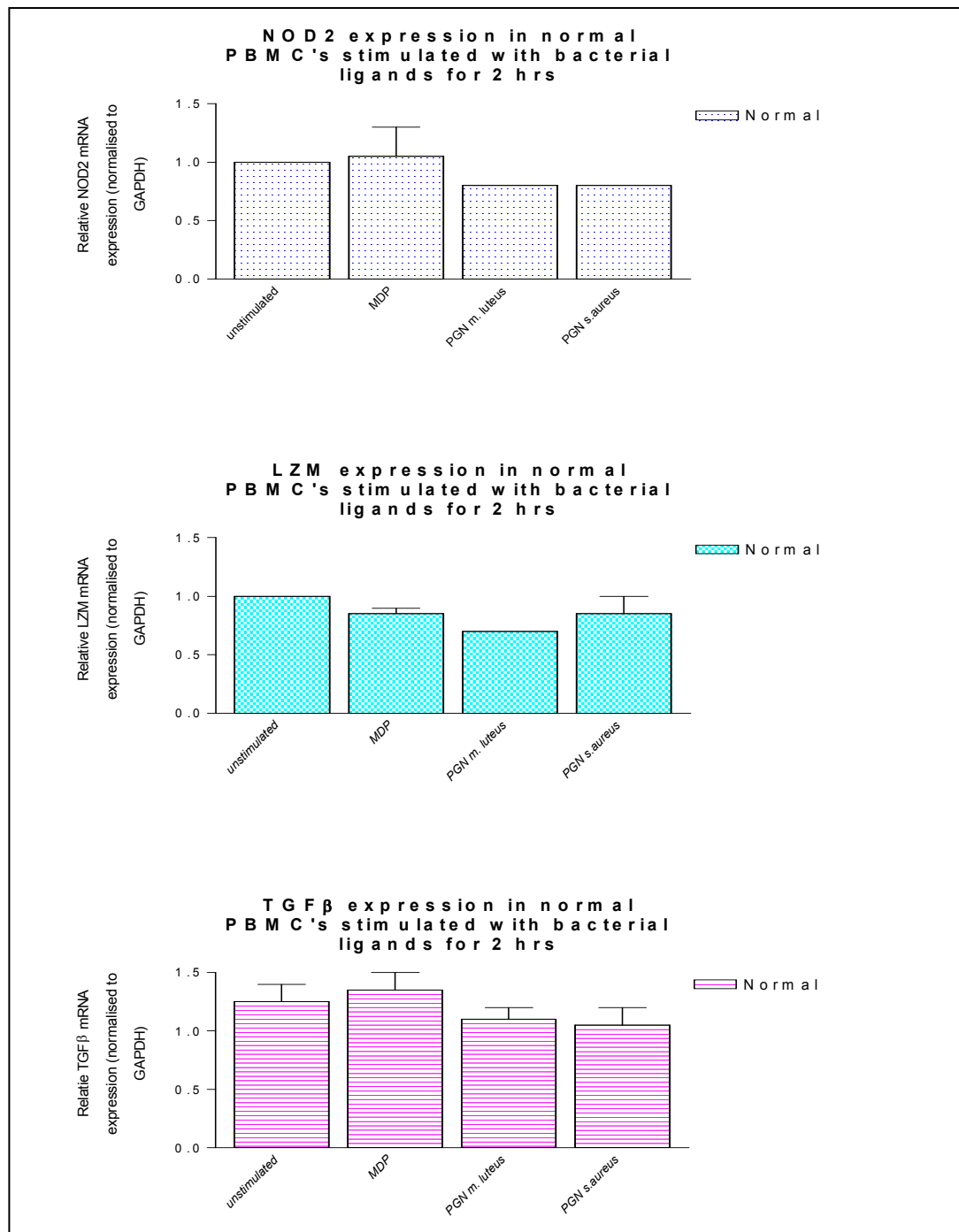


Figure 3.2-Stimulation of peripheral blood monocytes by bacterial ligands: NOD2, LZM & TGF β expression

3.2 Bacterial Stimulation of PBMC - Dose Response

3.2.1 Lipopolysaccharide (LPS)

The doses of bacterial ligands to be studied were first optimised. Lipopolysaccharide (LPS) is a component of the outer membrane of Gram negative bacteria and specific Gram positive bacteria (*Listeria monocytogenes*) which contributes to the bacteria's structural integrity. LPS is also an endotoxin known to activate the innate immune system through Toll like receptor 4. Fresh PBMC were isolated from healthy volunteers as previously described and concurrently stimulated in serum free media for 2 hours with Lipopolysaccharide (LPS) at one of the following doses -1ng/ml, 10ng/ml, 50ng/ml, 100ng/ml or 200ng/ml. After extraction of RNA and cDNA synthesis, quantitative real time PCR was performed looking at TNF α , IL-1 β and IL-8 cytokine expression. Results were normalised for GAPDH expression

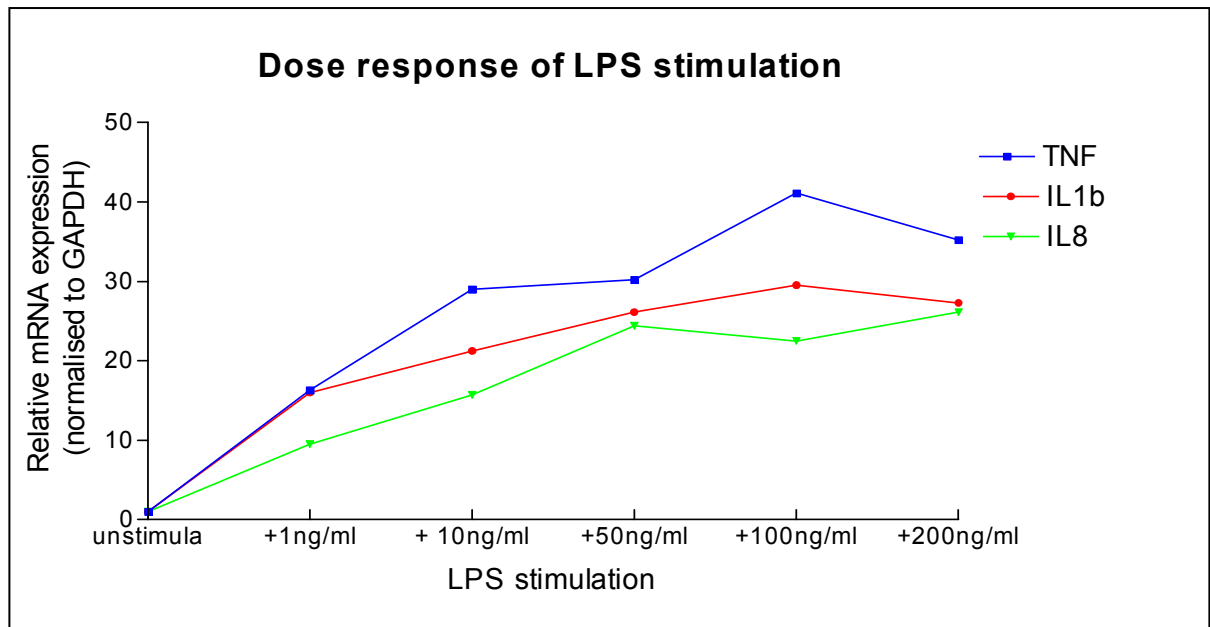


Figure 3.3 – Dose response to LPS stimulation, TNF α , IL1 β , IL8 expression

There is a linear increase in response to LPS from 1ng/ml to 100ng/ml where the effect is maximal causing a 41.1 fold induction in TNF α expression and 29.5 and 22.5 fold induction in terms of IL-1 β & IL-8 respectively. The effect plateaued on any further increase in LPS. It was entirely on the basis of the results shown above in the experiments I had performed in the laboratory, that I thus elected to use 100ng/ml LPS for the further experiments. (Figure 3.3)

3.2.2 Peptidoglycan (PGN)

Peptidoglycan (PGN) as discussed previously is an essential component of virtually all bacteria that provides rigidity and structure. The amount, location and specific composition of PGN varies between species. Responses to Peptidoglycan (PGN) from the following species, *micrococcus luteus* and *staphylococcal aureus* and sonicated *mycobacterium vaccae* were examined. They were concurrently stimulated for 2 hours at the following doses - 100ng/ml, 1µg/ml, 10µg/ml, 20µg/ml and 30µg/ml and then RNA extracted and analysed as previously described. Figure 3.4

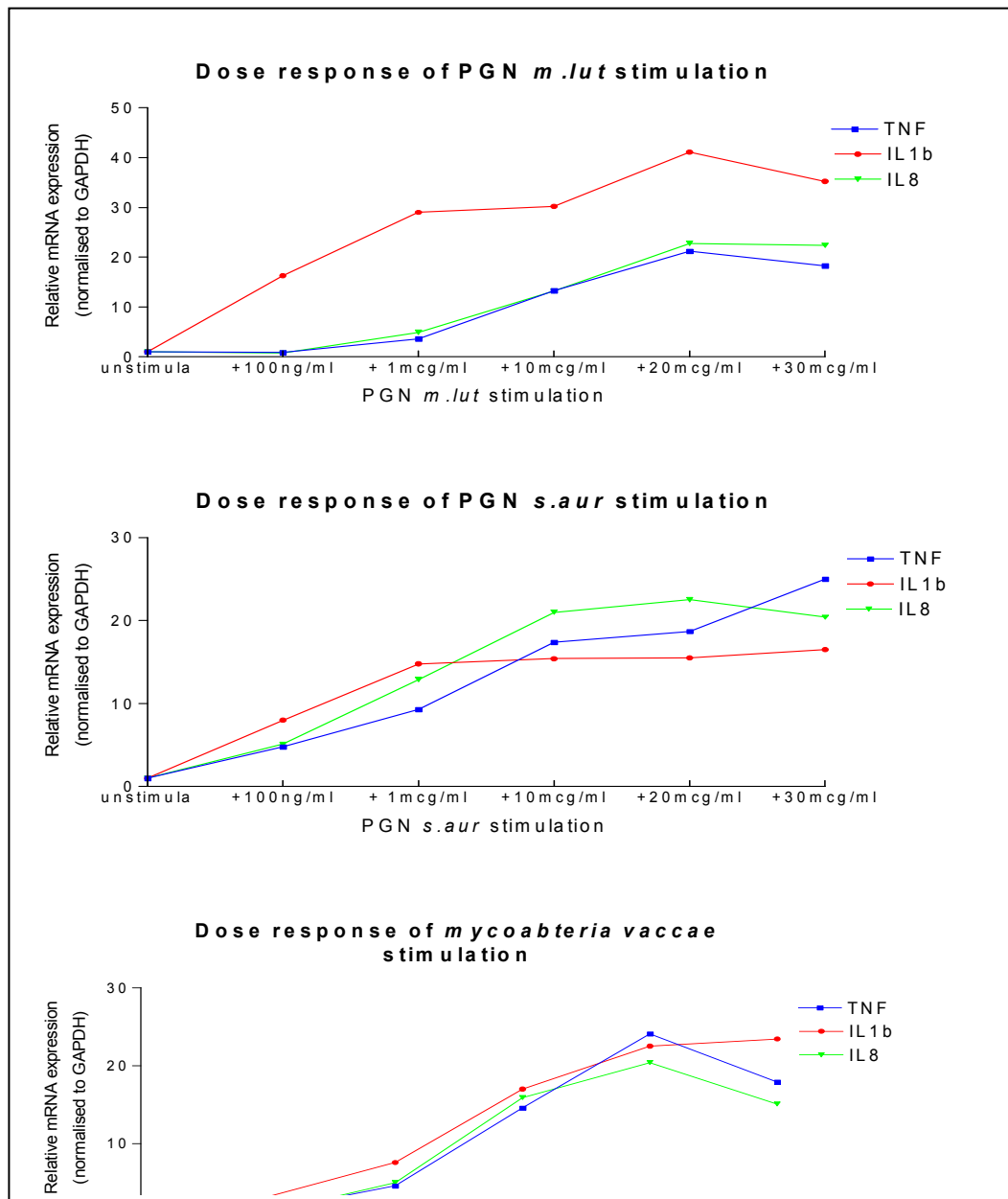


Figure 3.4 – Dose response to PGN stimulation, TNF α , IL1 β & IL8 expression

There is a maximum response in terms of TNF α , IL-1 β and IL-8 expression to PGN at 20 μ g/ml. The response was consistent across PGN from each of the 3 species studied with a mean of 23.2 fold increases in the 3 proinflammatory cytokines studied.

3.2.3 Muramyl dipeptide (MDP)

Fresh PBMC were isolated from healthy volunteers and again concurrently stimulated in serum free media for 2 hours with Muramyldipeptide at the following doses - 100ng/ml, 1 μ g/ml, 10 μ g/ml, 20 μ g/ml and 30 μ g/ml. After extraction of RNA and cDNA synthesis, quantitative real time PCR was performed looking at cytokine expression. Results were normalised for GAPDH expression and are shown in Figure 3.5

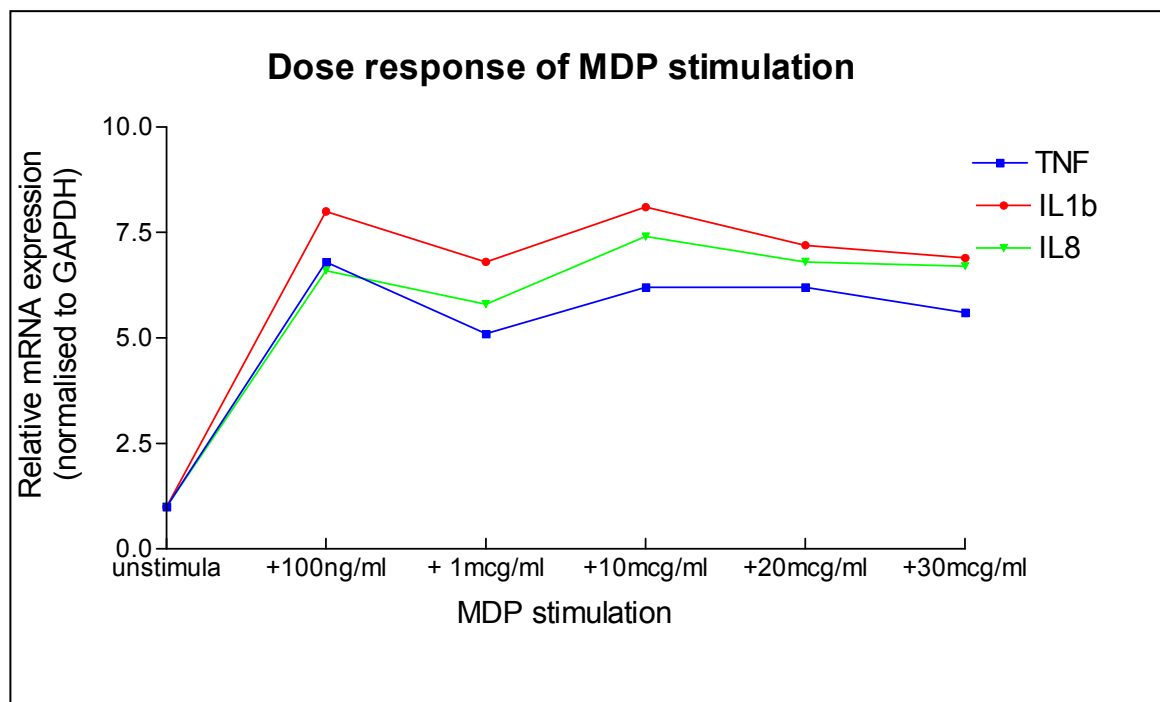


Figure 3.5 – Dose response to MDP stimulation, TNF α , IL1 β , & IL8 expression

MDP at 100ng/ml caused a mean 7.1 fold induction in TNF α , IL-1 β & IL-8 expression. Any further increase in MDP dose did not cause a resulting increase in cytokine expression.

3.2.4 Bacterial Stimulation of PBMC - Length of Stimulation

To establish the time at which the maximum response from bacterial stimuli is seen fresh PBMC were again isolated from healthy volunteers and stimulated with MDP, PGN and LPS. Cells were harvested at 1 hour, 2 hours, 4 hours and 6 hours. After extraction of RNA and cDNA synthesis, quantitative real time PCR was performed looking at $\text{TNF}\alpha$ and $\text{IL-1}\beta$ cytokine expression. Results were normalised for GAPDH expression. (Figure 3.6)

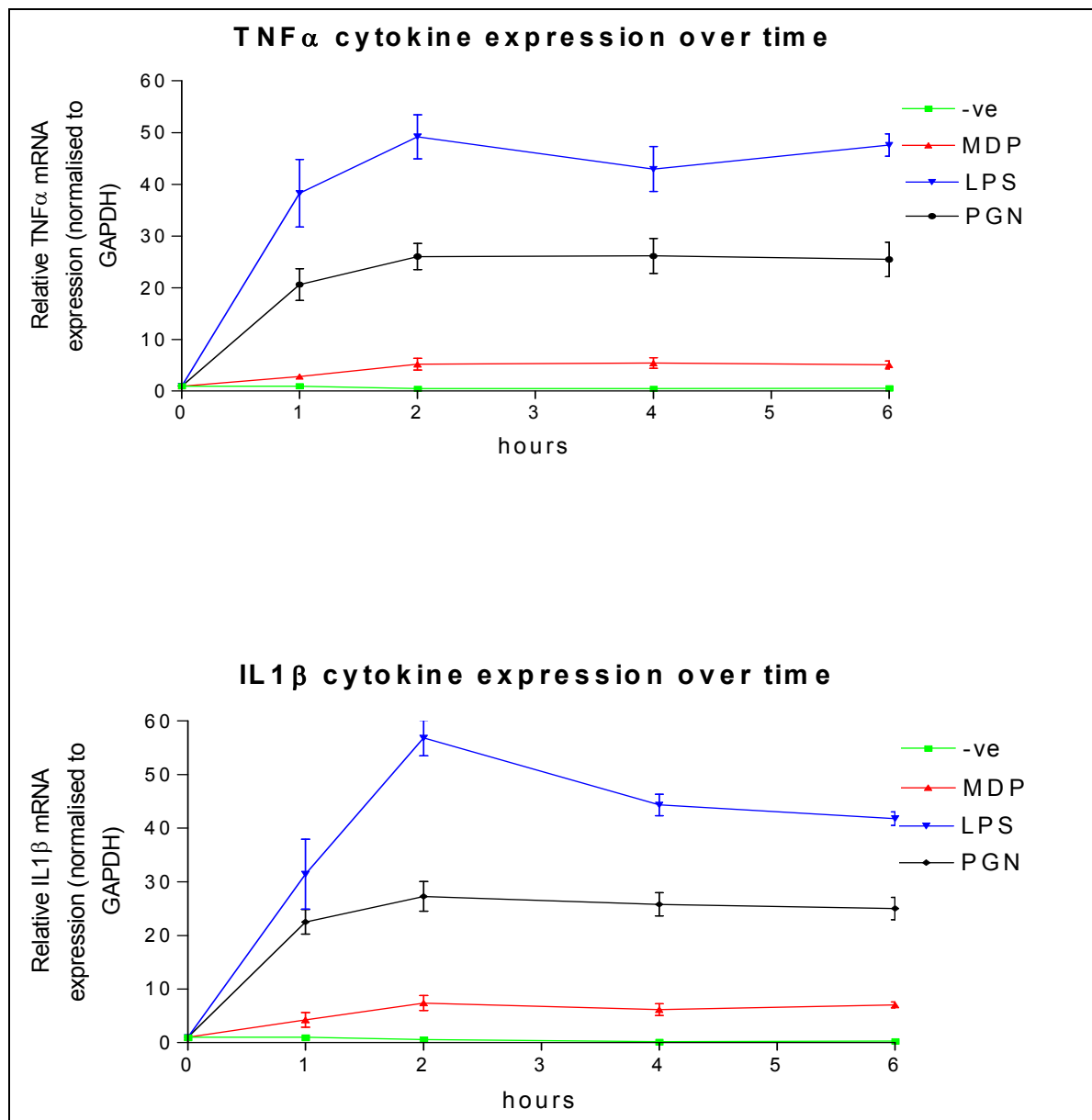


Figure 3.6 – PBMC response to MDP, PGN & LPS stimulation over time in terms of $\text{TNF}\alpha$ and $\text{IL1}\beta$ expression

This demonstrated that LPS was a potent inducer of inflammatory cytokine response and by two hours this response was maximal causing a mean 49.2 fold induction in $\text{TNF}\alpha$ and 56.8 fold induction in $\text{IL-1}\beta$. There was no further increase in induction and a plateauing effect to 6 hours. PGN and MDP caused a more modest induction in $\text{TNF}\alpha$ & $\text{IL1}\beta$ expression but this was also maximal at 2 hours. 2 hours of bacterial stimulation was therefore the time point used in future experiments.

3.3 Healthy control PBMC response to bacterial stimuli

The response in normal PBMC from healthy volunteers to various stimuli was then examined more closely. Blood was obtained from 12 healthy volunteers and the PBMC then extracted. The NOD2 status on these healthy controls was not examined.

Freshly isolated PBMC were stimulated in serum free media for 2 hours with either

1. Muramyl dipeptide (20 μ g/ml)
2. Peptidoglycan (PGN) *m.luteus* (20 μ g/ml)
3. Lipopolysaccharide (LPS) (100ng/ml)

After extraction of RNA and cDNA synthesis, quantitative real time PCR was performed looking at cytokine expression and results were normalised to expression of the house-keeping gene, GAPDH.

In freshly isolated PBMC from 12 healthy volunteers MDP causes only a mean 3.11 fold increase in TNF α expression (SEM 0.40) whilst PGN induces a mean 30.19 fold increase (SEM 0.40) whilst LPS is the most potent inducer of TNF expression with a 54.04 fold increase in expression (SEM-9.40). Figure 3.7

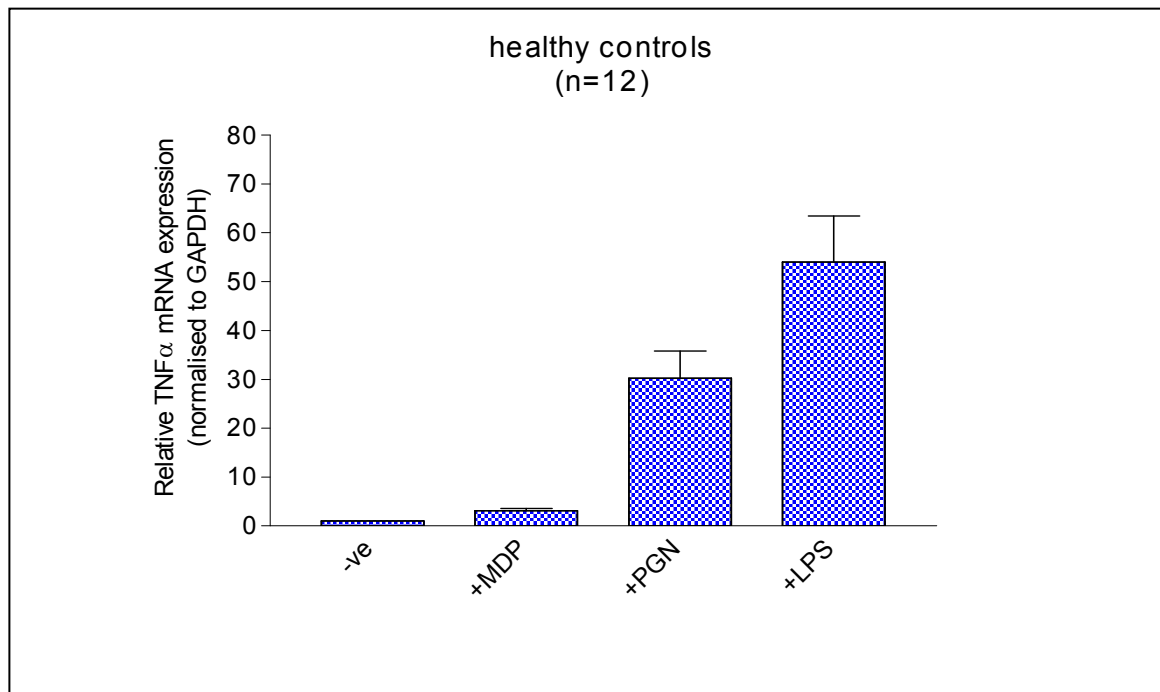


Figure 3.7-Stimulation of peripheral blood monocytes by bacterial ligands: TNF α expression

I then went on to examine interleukin-1 β (IL-1 β) and interleukin-8 (IL-8) expression, cytokines involved in the immediate innate immune response. Figure 3.8

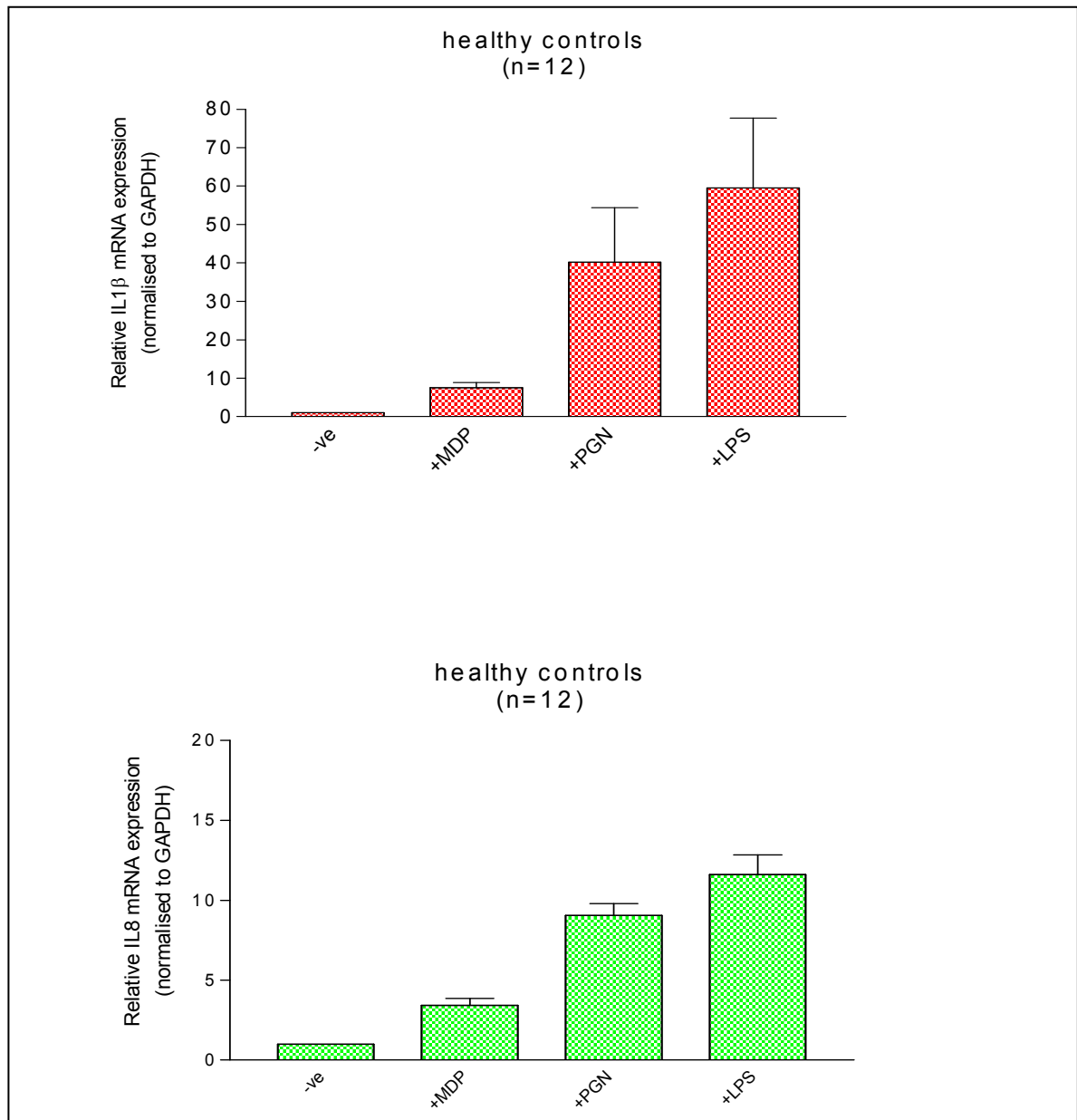


Figure 3.8 – Stimulation of peripheral blood monocytes by bacterial ligands: IL-1 β and IL-8 expression

These cytokines demonstrated the same stimulatory hierarchy with MDP being the weakest stimulant of cytokine expression and LPS being the most powerful inducer causing a 59.48 fold increase in IL1- β expression (SEM 18.21). Although the same hierarchy is seen in IL-8 expression with MDP being the weakest, LPS the most potent and PGN intermediate, the

relative fold induction is less pronounced with only an 11.6 fold increase in response to LPS stimulation and so following this I focussed primarily on TNF α and IL-1 β expression.

3.4 Clinical Characteristics of Crohn's patients studied

Having established the effect of bacteria on healthy control PBMC I then wanted to compare the effect of bacterial stimulation on PBMC in patients with Crohn's disease who were wild type, heterozygous or homozygous for the common disease causing mutations in the NOD2 gene. 28 patients with Crohn's disease were recruited from the Inflammatory Bowel Disease clinic at the Royal Free Hospital, London between January 2004 and July 2005. There is potential selection bias here as only 5 NOD2 homozygotes have been identified thus far from the IBD clinic and these were all asked to participate. There was a larger pool of patients from which to select the heterozygotes or wild type patients from and this was done on the basis of whom was attending the weekly clinic. This however increases the possibility that patients who were clinically well were less likely to be attending the clinic, thus introducing bias.

Clinical information including Vienna Classification, IBD treatment and disease activity at the time of venesection was collected and the results are shown in Tables 3.1, 3.2 and 3.3 respectively. Twelve patients were wild type for the common disease causing NOD2 mutations, 7 male and 5 female with a mean age of 45.8yrs. There were 11 patients who were heterozygous for the common disease causing mutations in the NOD2 gene -7 male and 4 female with a mean age of 41.0 yrs and 5 who were homozygous or compound heterozygotes, 2 male and 3 female with a mean age of 38.4yrs. The overall mean age of the Crohn's patients studied was 42.6yrs (range 14-82 yrs) and the sex ratio was 1.33:1 (M:F) There was no significant difference in age, sex or disease classification between groups. Other differences between the cohorts are discussed below.

3.4.1 Disease Activity

In terms of disease activity at time of venesection 2 out of 12 (16.7%) of the patients wild type for the common NOD2 mutations had active disease whereas 4 of the 11 heterozygotes

(36.4%) and 3 out of the 5 homozygotes (60%) patients had active disease at time of the venesection. This may be of significance.

3.4.2 Use of immunomodulator treatment

Use of immunomodulator treatment including azathioprine, Mercaptopurine or Methotrexate was common in all groups with a total of 12 of the 28 (42.9%) being on one of these second line agents. Use was highest proportionally amongst the wild type group where 7 of the 12 (58.3%) were currently taking a thiopurine or Methotrexate. 3 of the 11 heterozygotes (27.3%) and 2 of the 5 (40%) homozygotes were using a thiopurine or Methotrexate at the time of venesection. The dose of oral thiopurine ranged between 75mg and 200mg but no data are available assessing the efficacy of the treatment in terms of Azathioprine metabolites.

3.4.3 Infliximab use

There were no current users of the TNF antibody Infliximab in either the wild type or heterozygote group but 2 from each group (16.7% and 18.2% respectively) had previously (at least 12/12 ago) received it. Amongst the homozygote group there was only one patient (20%) who had received Infliximab and was receiving ongoing maintenance treatment every 8 weeks at the time of venesection.

3.4.4 Smoking Status

The rate of smoking in our cohort was lower than expected with only 4 of the 28 (14.3%) being current smokers. These were again equally distributed with 2 each amongst the wild type and heterozygote groups. There were a further 2 ex-smokers, (defined as not smoked for over 6 months) one wild type and one heterozygote for the common disease causing mutations in the NOD2 gene. None of the patients who were homozygous for the common disease causing mutations in the NOD2 gene were either current or ex-smokers.

Table 3.1 Clinical Characteristics of Patients Studied – NOD2 +/-

-----At time of venesection-----									
Subject	NOD2 status	Sex	Age	Comorbidity	Smoker	IBD Drugs	Disease Activity	Infliximab Use	Disease Classification ¹
MM	wt/wt	Male	41	Nil	Smoker	Azathioprine 100mg OD, Pentasa 2g BD	Active	Never	A1, L3, B2
OW	wt/wt	Male	26	Nil	Smoker	Pentasa 1g BD	Quiescent	Never	A1, L3, B1
DE	wt/wt	Female	21	Nil	NS	Asacol 1200mg BD Azathioprine 150 mg OD	Quiescent	Never	A1, L2, B1
IB	wt/wt	Female	49	Nil	NS	Methotrexate 15mg o/wk, Pentasa 1g BD, Pred 7mg	Active	Previous (2002)	A1, L3, B1
RB	wt/wt	Male	64	Nil	NS	Azathioprine 100mg OD Pentasa 1g BD	Quiescent	Never	A2, L4, B1
AB	wt/wt	Male	44	Nil	NS	Mercaptopurine 50mg OD	Quiescent	Never	A1, L4, B2
JM	wt/wt	Female	76	IHD, NIDDM Sarcoid	Ex smoker	Nil	Quiescent	Never	A2, L4, B1
RM	wt/wt	Male	45	Nil	NS	Azathioprine 200mg OD, Pentasa 1g BD	Quiescent	Previous (2000)	A1, L3, B2
MC	wt/wt	Female	62	Nil	NS	Nil	Quiescent	Never	A2, L1, B1
ES	wt/wt	Female		Nil	NS	Nil	Quiescent	Never	A1, L2, B1
DI	wt/wt	Male	21	Nil	NS	Pentasa 1g BD	Quiescent	Never	A1, L2, B1
PU	wt/wt	Male	59	IHD	NS	Mercaptopurine 75mg OD, Asacol 800mg BD	Quiescent	Never	A2, L3, B1

¹ According to Vienna Classification – see Appendix B.

Table 3.2 Clinical Characteristics of Patients Studied – NOD2+/-

-----At time of venesection-----									
Subject	NOD2 status	Sex	Age	Comorbidity	Smoker	IBD Drugs	Disease Activity	Infliximab Use	Disease Classification
AC	13+/-	Male	31	Nil	NS	Pentasa 1g BD	Active	Never	A1, L3, B1
LOS	12+/-	Female	82	Hypertension	NS	Nil	Quiescent	Never	A1, L3, B3
SO	8+/-	Male	19	Gender Reassignment	NS	Azathioprine 150mg OD, Pentasa 1g BD	Quiescent	Never	A1, L3, B1
SM	12+/-	Female	40	Nil	Ex smoker	Azathioprine 100mg OD	Active	Previous (2000)	A1, L3, B3
DH	13+/-	Male	27	Nil	NS	Pentasa 1g BD	Quiescent	Never	A1, L3, B3
SS	8+/-	Male	49	Nil	NS	Nil	Quiescent	Never	A2, L1, B1
HC	8+/-	Female	21	Nil	NS	Asacol 1200mgBD	Quiescent	Never	A1, L3, B1
MG	8+/-	Male	70	Nil	NS	Pentasa 1g BD	Quiescent	Never	A2, L3, B1
LS	8+/-	Female	19	Nil	Smoker	Azathioprine 100mg OD, Budesonide 9mg OD	Active	Previous	A1, L4, B3
AD	13+/-	Male	19	Nil	Smoker	Nil	Quiescent	Never	A1, L1, B1
FP	8+/-	Male	74	Hypertension	NS	Pentasa 1g BD	Active	Never	A2, L1, B1

¹ According to Vienna Classification – see Appendix B

Table 3.3 Clinical Characteristics of Patients Studied – NOD2 -/-

-----At time of venesection-----									
Subject	NOD2 status	Sex	Age	Comorbidity	Smoker	IBD Drugs	Disease Activity	Infliximab Use	Disease Classification ¹
LC	12-/8-	Male	72	Chronic Lymphocytic leukaemia – stage A	NS	Nil	Quiescent	Never	A2, L4, B1
PB	13-/-	Female	29	Nil	NS	Azathioprine 100mg OD, Pentasa 1g BD	Active	Previous	A1, L4, B3
AE	8-/-	Male	14	Nil	NS	Pentasa 2g BD	Active	Never	A1, L3, B1
SS	12-/8-	Female	50	Nil	NS	Methotrexate 10mg /wk, Pentasa 500mg TDS	Active	Never	A1, L4, B3
JH	13-/-	Female	27	Nil	NS	Infliximab Maintenance	Active	Current	A1, L3, B1

¹ According to Vienna Classification – see Appendix B

3.5 Bacterial stimulation of PBMC in Patients with Crohn's Disease

Fresh PBMC were collected and stimulated with bacterial ligands as described in section 3.3. The results for TNF α and IL-1 β cytokine expression were determined and results for TNF α shown in Figure 3.9 in comparison to healthy controls. In patients with Crohn's Disease (n=28), PBMC respond to bacterial stimuli with the same hierarchical activity as in healthy individuals. (IL-1 β data in Figure 3.11) LPS was again the most potent stimulator causing a mean 46.97 fold increase in TNF α expression (SEM 5.75). This compares to 54.04 fold induction in PBMC from healthy controls. PGN caused a 29.63 fold induction in TNF α expression (SEM 5.51) which is similar to healthy controls (30.19 fold increase) MDP was the weakest stimulator causing a mean of 2.64 fold induction (SEM 0.37) in TNF α from PBMC from Crohn's patients compared to 3.11 in PBMC from healthy controls.

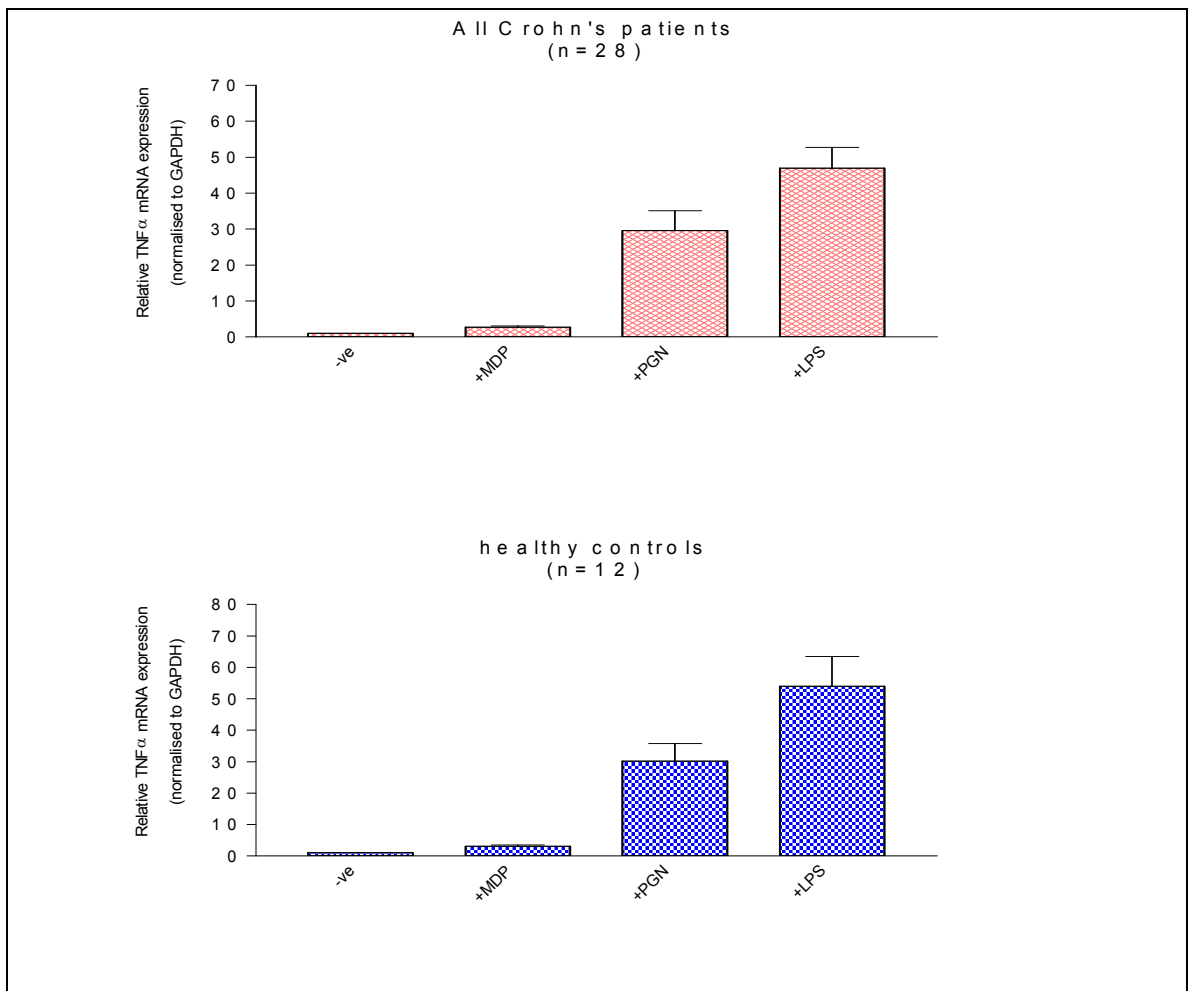


Figure 3.9 - Response of PBMC to bacterial stimuli from Crohn's patients compared to healthy controls -TNF α expression

3.6 Bacterial stimulation of PBMC across NOD2 genotypes

I then went onto examine whether the NOD2 genotype affected how PBMC responded to bacterial stimulation. The results are shown in Figure 3.10 for TNF α expression and in Figure 3.11 for IL-1 β gene expression. The same hierarchical activity was seen in all patients regardless of the NOD2 genotypes. LPS was the most potent inducer causing a 54 fold, 41 fold, 55.6 fold or 41.54 fold increase in TNF α expression in healthy controls, wild type, heterozygotes and homozygotes respectively. MDP was a weak stimulant inducing between a 2.58 to 3.45 fold increase in TNF α expression.

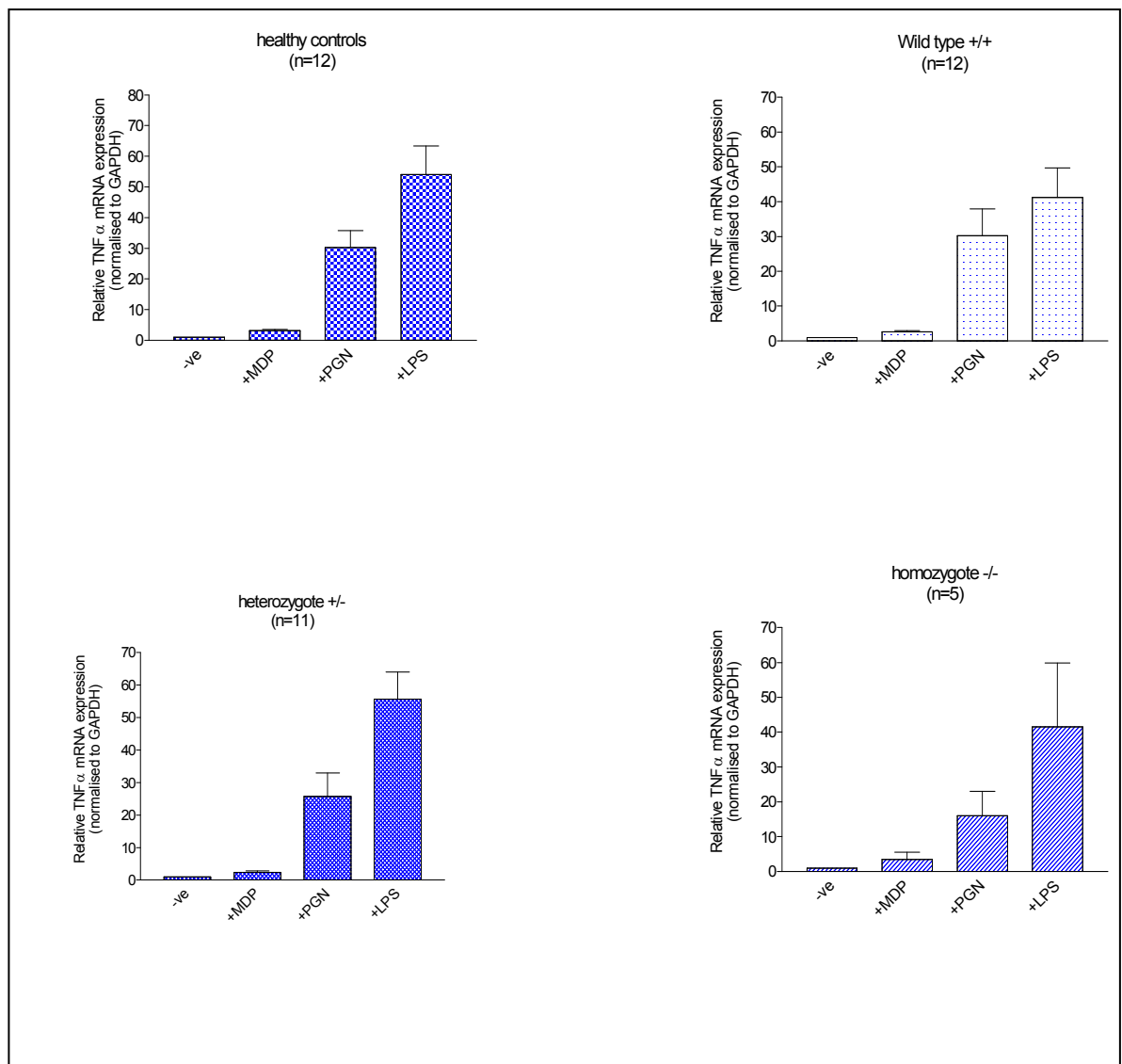


Figure 3.10- Response of PBMC to bacterial stimuli across the NOD2 genotypes - TNF α expression

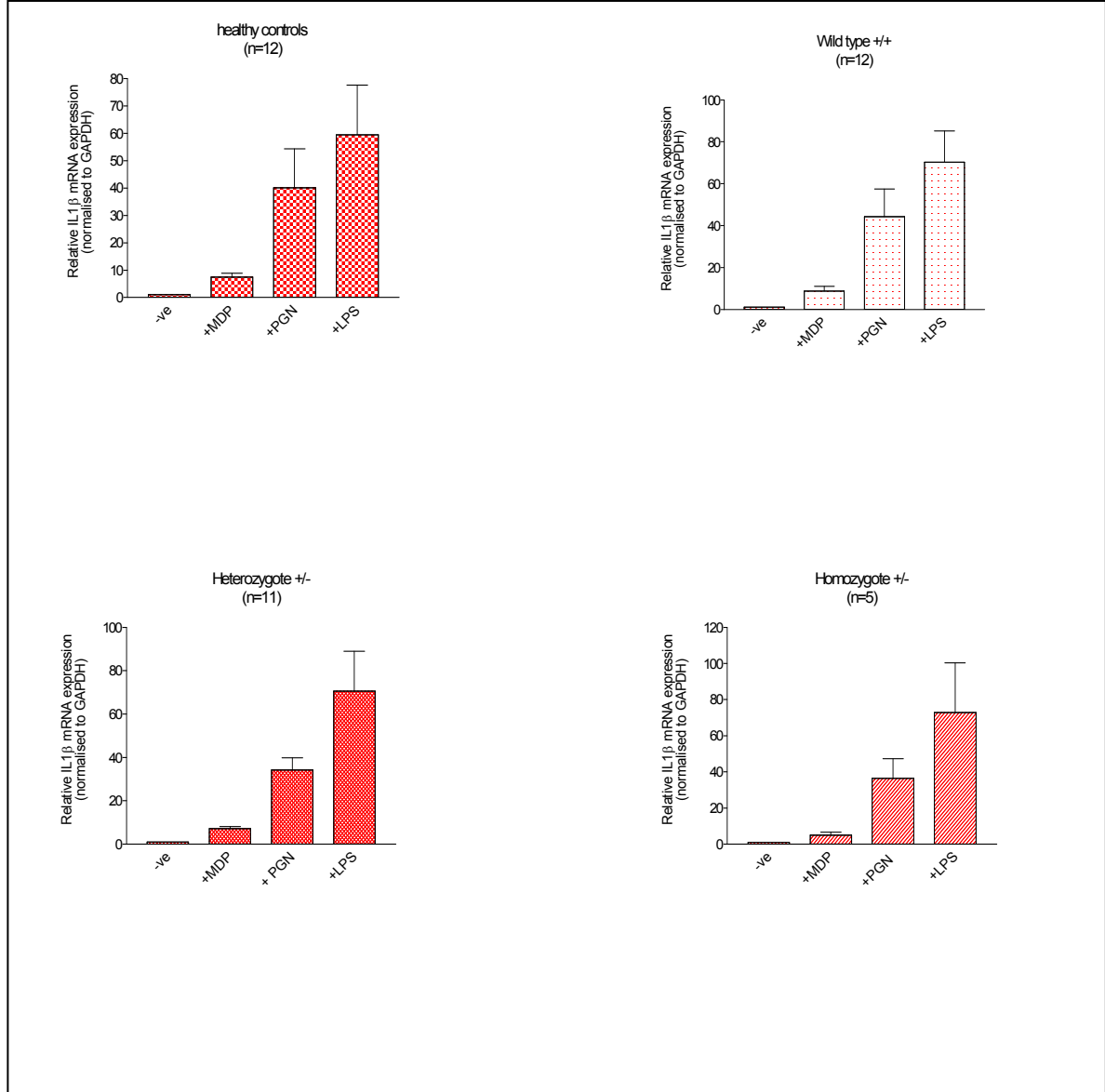


Figure 3.11 – Response of PBMC to bacterial stimuli across the NOD2 genotypes – IL1 β expression

This hierarchical effect was the same when examining IL-1 β expression. MDP was a weak inducer across all the genotypes, ranging between a mean of 4.9 and 8.83 induction in IL-1 β expression. LPS was the most potent inducer again causing between a 59.47 and 72.74 fold induction in IL-1 β expression. (Figure 3.11)

To help determine whether my healthy samples represent a normal Gaussian Distribution population I performed a Kolmogorov-Smirnov (KS) test. When examining results from LPS, PGN and MDP stimulated PBMC the P value obtained was >0.10 in each thereby passing the normality test. (KS distance 0.118) It is hard to verify completely however that the samples are normally distributed due to the small sample size (n=12).

3.6.1 Stimulation of PBMC by LPS across genotypes

To further clarify the effect of bacterial stimulation I then compared the effect of each of the bacterial ligands on PBMC from healthy controls and those Crohn's patients carrying 0, 1 or 2 NOD2 disease causing mutations. LPS, an amphiphilic material in the outer membrane of both gram positive and gram negative bacteria is a well known activator of macrophages and is sensed through TLR 4.

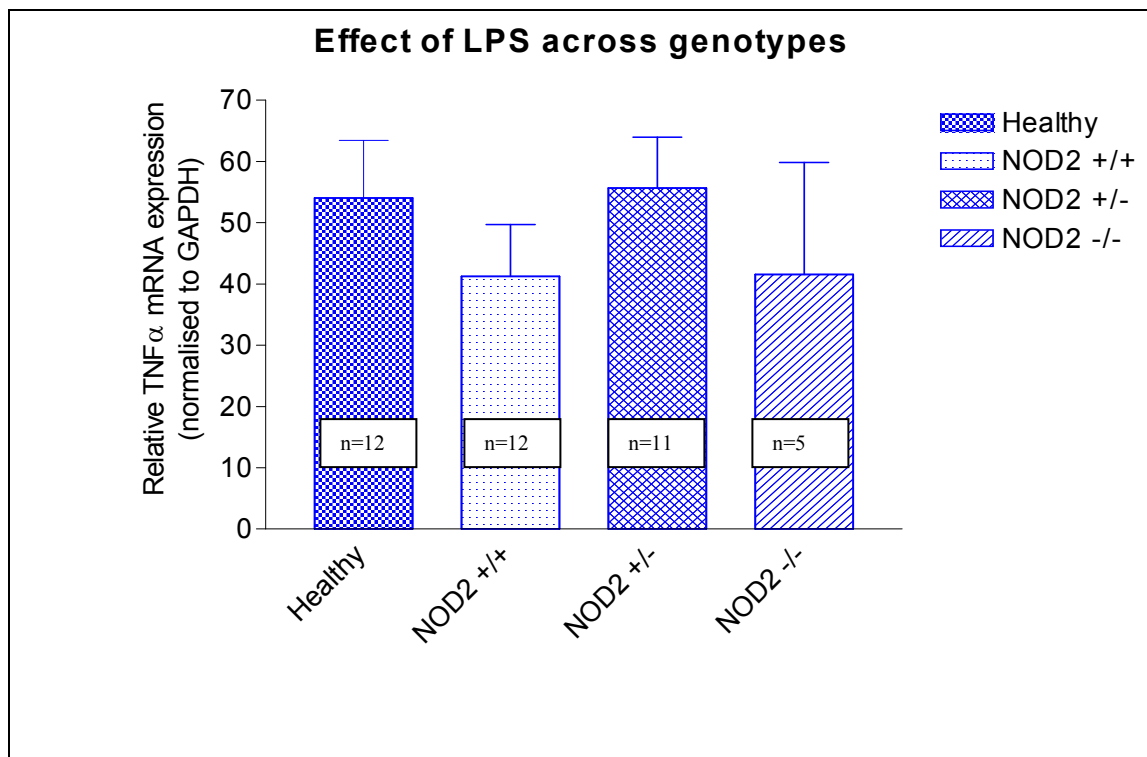


Figure 3.12 – Effect of LPS on PBMC from healthy controls & across NOD2 genotypes

Toll like receptors (TLR's) are a family of pattern recognition receptors that sense motifs found in microbial pathogens and initiate immune defense against such pathogens. TLR are found in PBMC but are membrane bound in contrast to NOD2 which lies within the cytoplasm. This showed (Figure 3.12) that LPS caused a mean 49.08 fold induction in TNF α expression (SEM 4.87) in PBMC and there was no significant difference in the cytokine response between patients and healthy controls and across the NOD2 genotypes. This was as expected as LPS is sensed through TLR 4 which is present in all PBMC and therefore the NOD2 status should not be relevant.

3.6.2 Stimulation of PBMC by PGN across genotypes

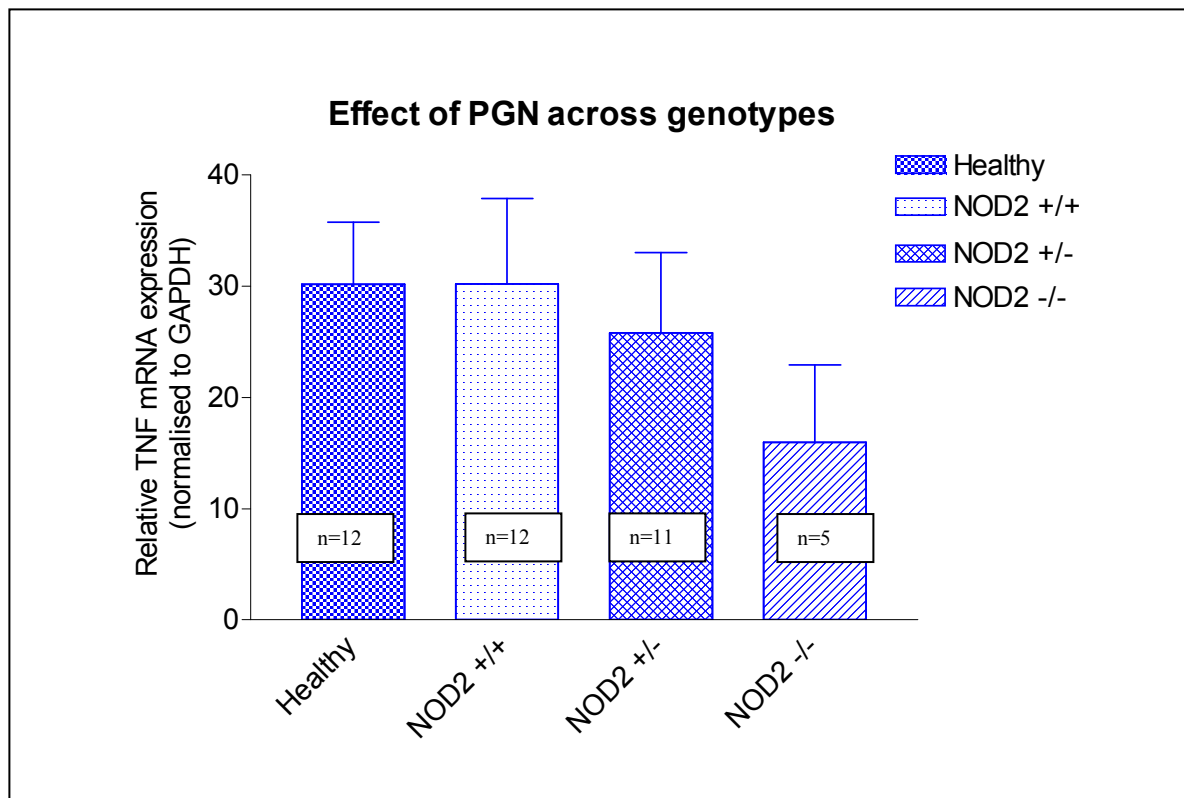


Figure 3.13 – Effect of PGN on PBMC from healthy controls & across NOD2 genotypes

PGN induced a mean 30.19 fold increase in TNF α expression in healthy controls and 30.21 and 25.73 fold increase in TNF α expression in PBMC from wild type and heterozygotes. In homozygotes PGN still caused an increase in TNF α expression but this was relatively reduced to 15.97 fold increase. This does not reach statistical significance but a possible explanation for this is that PGN is sensed through both toll like receptor 2 which should be similar in PBMC from all genotypes and also through its breakdown product MDP which may be reduced in the PBMC carrying two NOD2 mutations.

3.6.3 Stimulation of PBMC by MDP across genotypes

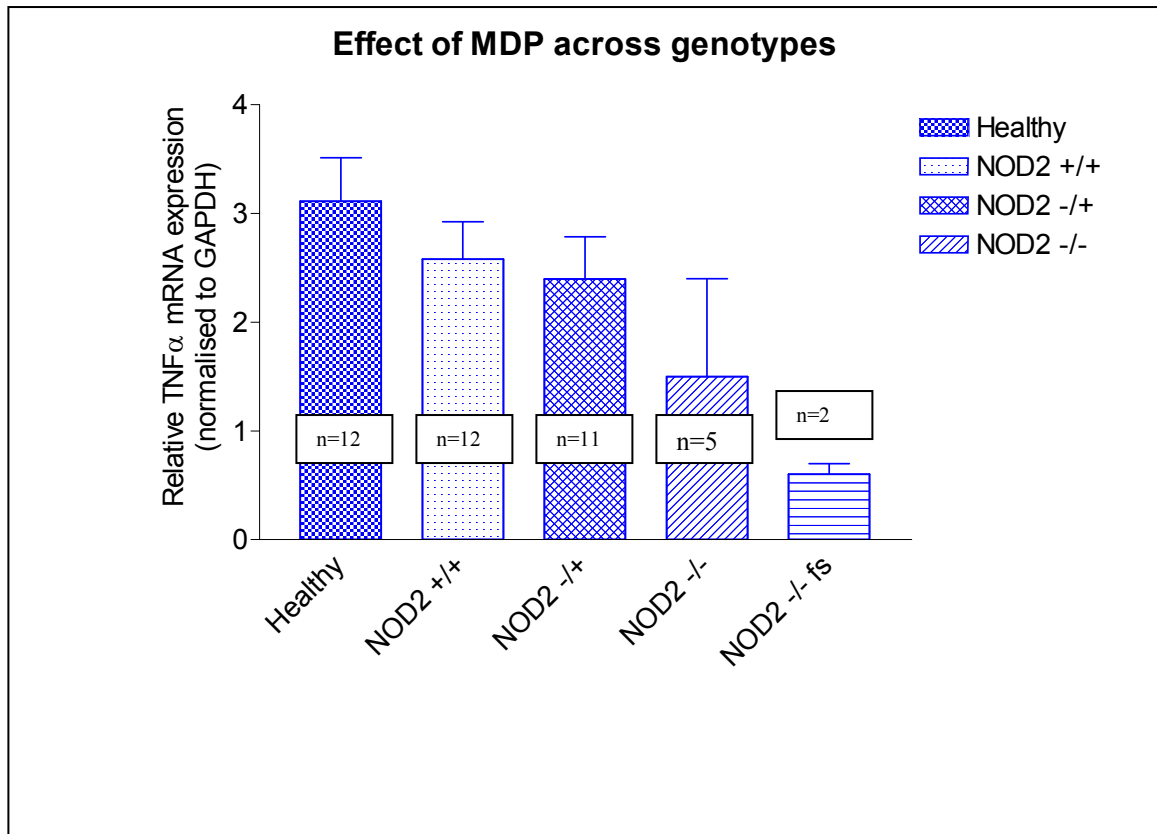


Figure 3.14 – Stimulation with MDP – Response across NOD2 genotypes

Examining the response to MDP shows that the response is minimal even in healthy PBMC causing only a 3.11 fold induction in TNF α expression. The effect of MDP on PBMC wild type and heterozygote for the NOD2 mutations is reduced -2.58 fold and 2.45 fold respectively. When studying the homozygotes the response is further reduced but not at a statistically significant level. The response of the PBMC who were homozygote for the frameshift mutations (n=2) was then examined separately and this demonstrated a further reduced response to MDP. This compares with the previous published data (Li 2004, Ogura 2001, Inohara 2003) that demonstrated that the frameshift mutation caused reduced NF κ B activation.

3.6.4 TGF β , LZM and NOD2 expression in Crohn's PBMC stimulated with bacterial ligands

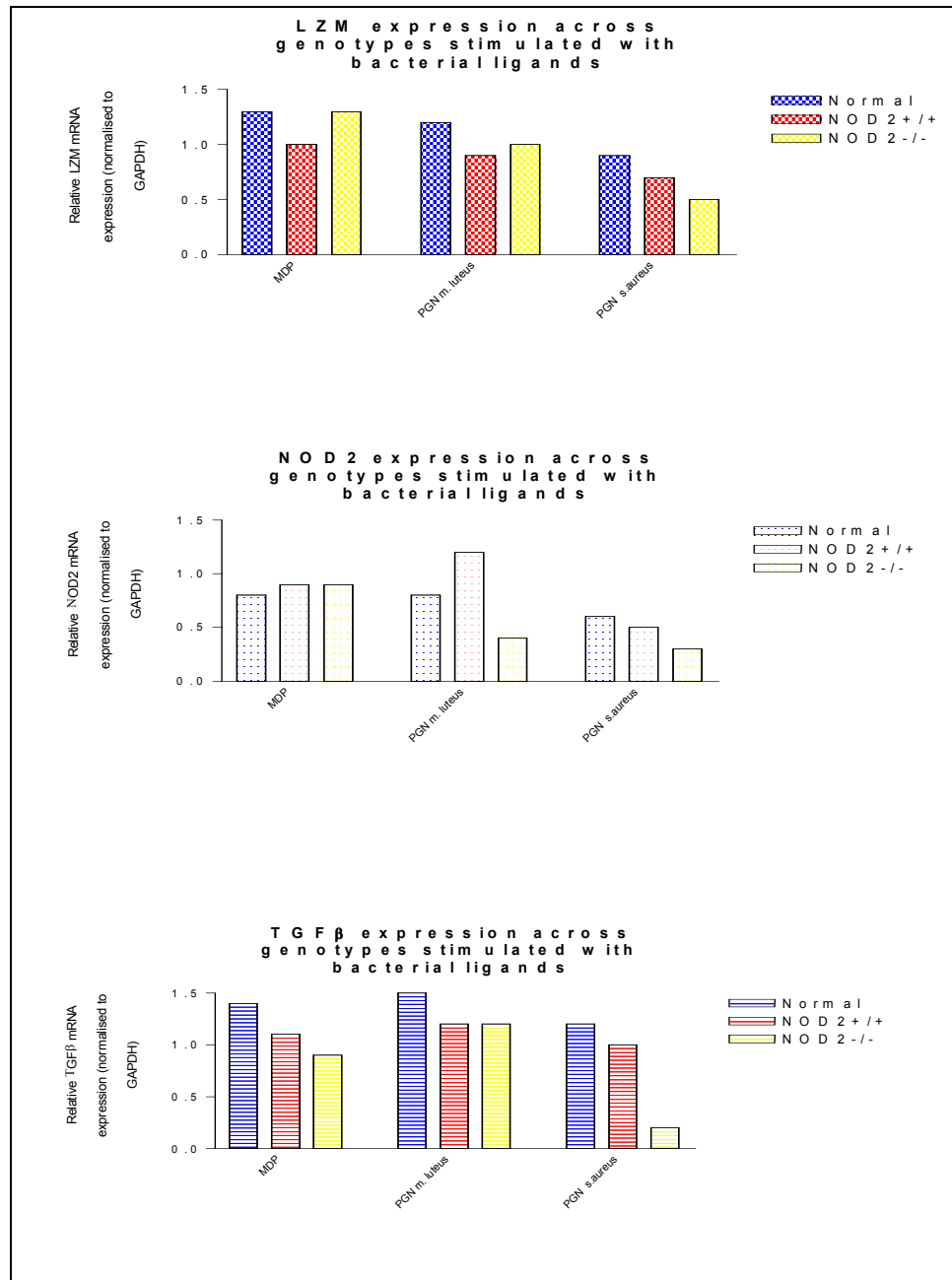


Figure 3.15 – NOD2, LZM and TGF β expression unchanged across genotypes

I previously demonstrated that normal PBMC from healthy volunteers when stimulated with bacteria did not respond by altering LZM, TGF β or NOD2 expression (Figure 3.2). I

therefore wanted to ensure that this was unchanged in PBMC from Crohn's patients with or without a NOD2 mutation. I therefore directly compared in a single experiment fresh PBMC from a healthy control, and two Crohn's patients who were wild type or homozygous for the common NOD2 disease causing mutations. PBMC were stimulated with MDP, PGN *m.lut* and PGN *s.aureus* for 2 hours and RNA extracted and cDNA synthesized as per protocol. LZM, TGF β and NOD2 expression remained unchanged in each of the PBMC regardless of stimulation. (Figure 3.15)

Contrary to these initial experiments Netea and colleagues (Netea 2004) subsequently published their work which also examined the cytokine response of PBMC to bacterial stimulation. They examined PBMC from 8 healthy controls and 8 patients with Crohn's disease (4 double frameshift mutation, 4 wild type) and demonstrated that the frameshift mutation resulted in defective release of the anti inflammatory cytokines TGF β and IL-10 rather than of the proinflammatory cytokine TNF α from cells stimulated with the TLR2 ligand PGN. They suggest that the reduced anti inflammatory cytokines production results in a pro inflammatory cytokine bias and they suggest this could be the basis of mechanism of NOD2 associated CD. These results have not been replicated.

3.7 Differential response across bacteria - Do mycobacteria depend on NOD2 for sensing?

Mycobacteria, particularly *Mycobacteria paratuberculosis* remains a putative, though controversial cause of Crohn's disease as discussed previously. (Chapter 1.3.4.2) Mutations in the *NOD2* gene are strongly associated with Crohn's disease. Furthermore an increased incidence of *Mycobacteria paratuberculosis* has been reported in patients with Crohn's Disease who carry *NOD2* gene mutations (Behr 2004). More recently an *in vitro* study suggests that NOD2 is an essential recognition molecule for *Mycobacterium tuberculosis* (MTB) and pronounced cellular activation is noted in NOD2-transfected cells that are stimulated with heat-killed MTB preparations. (Ferwerda 2005)

We therefore hypothesised whether mutant NOD2 proteins would influence cellular activation in response to mycobacterial antigen stimulation. We used two different strains of mycobacteria to assess whether different mycobacteria would illicit different response. Sonicated forms of *Mycobacterium vaccae* (MV) and *Mycobacterium tuberculosis* (MTB) were the mycobacteria species tested.

Blood was extracted from twelve Crohn's patients attending the Inflammatory Bowel Disease clinic at the Royal Free Hospital, London. Five patients were wild type for the common NOD2 mutations, four patients carried one disease causing NOD2 mutation (heterozygote) and three patients had two disease causing NOD2 mutations— one of whom was homozygous for the Leu1007fsinsC mutation. Fresh PBMC were isolated and prepared as previously described at a cell concentration of 4×10^6 cells / well in 6 well plates. They were then stimulated in serum free media for 2 hours with either:-

1. Muramyl dipeptide (20 µg/ml)
2. *Mycobacterium vaccae* (MV) (Strain NCTC 11659)
3. *Mycobacterium tuberculosis* (MTB) (Strain H37RV)
4. Lipopolysaccharide (LPS) (100 ng/ml)

After 2 hours incubation RNA was extracted, cDNA was synthesised, quantitative real time PCR was performed looking at cytokine expression and results were normalised to

expression of the house keeping gene GAPDH. The response to MDP and LPS was used as the experimental positive control.

3.7.1 Stimulation of PBMC by MDP across NOD2 genotypes

The response of PBMC to MDP stimulation is seen in Figure 3.16. As expected MDP was a weak stimulant causing a mean 4.5 fold increase in TNF α expression and 7.1 fold increase in IL-1 β expression. There was no significant difference across the NOD2 genotypes but as expected the patient homozygous for the Leu1007fsinsC mutation had a reduced response to MDP.

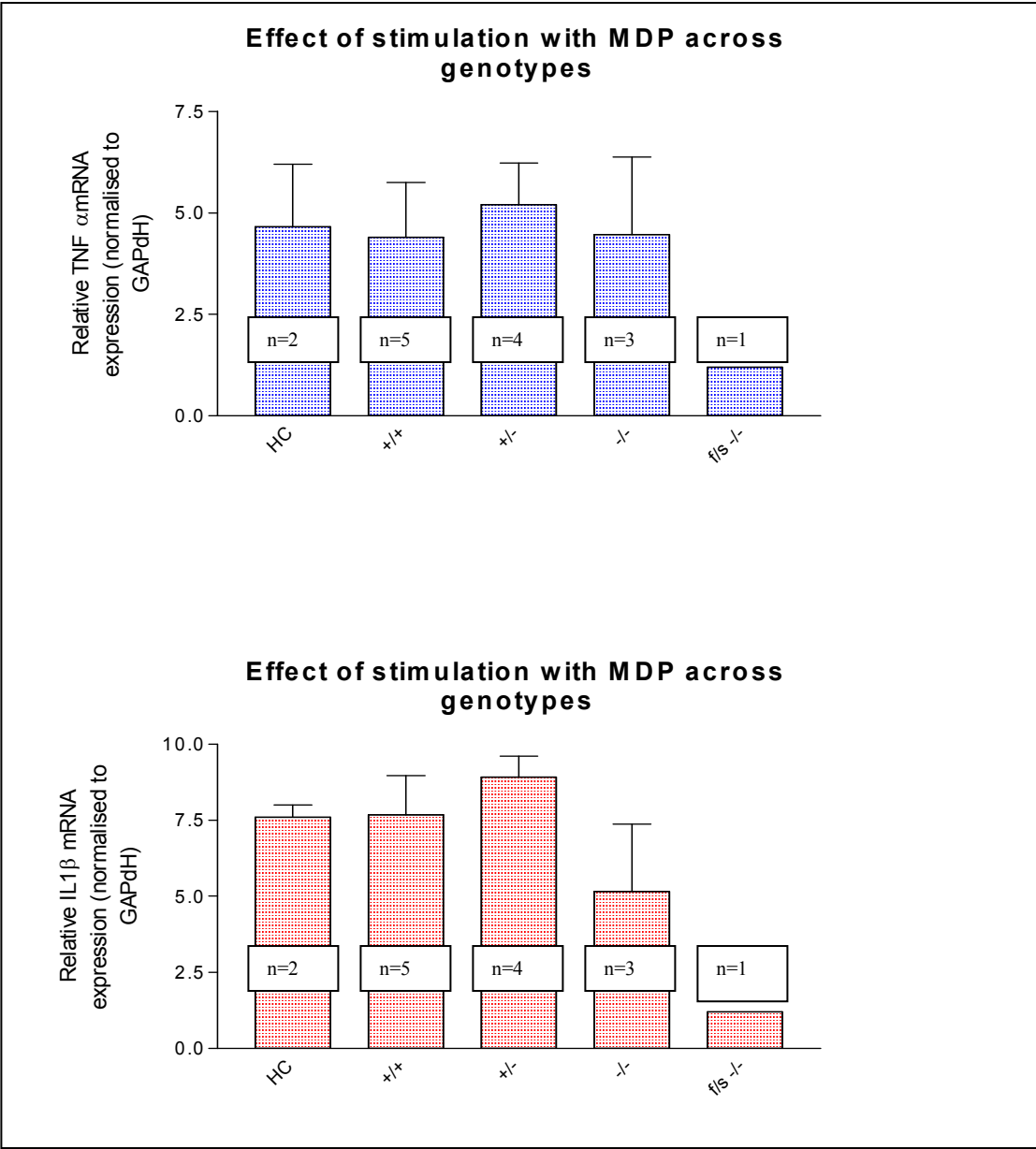


Figure 3.16 – TNF α and IL-1 β cytokine response to MDP stimulation by PBMC across genotypes

3.7.2 Effect of *Mycobacterium Tuberculosis* on PBMC across NOD2 genotypes

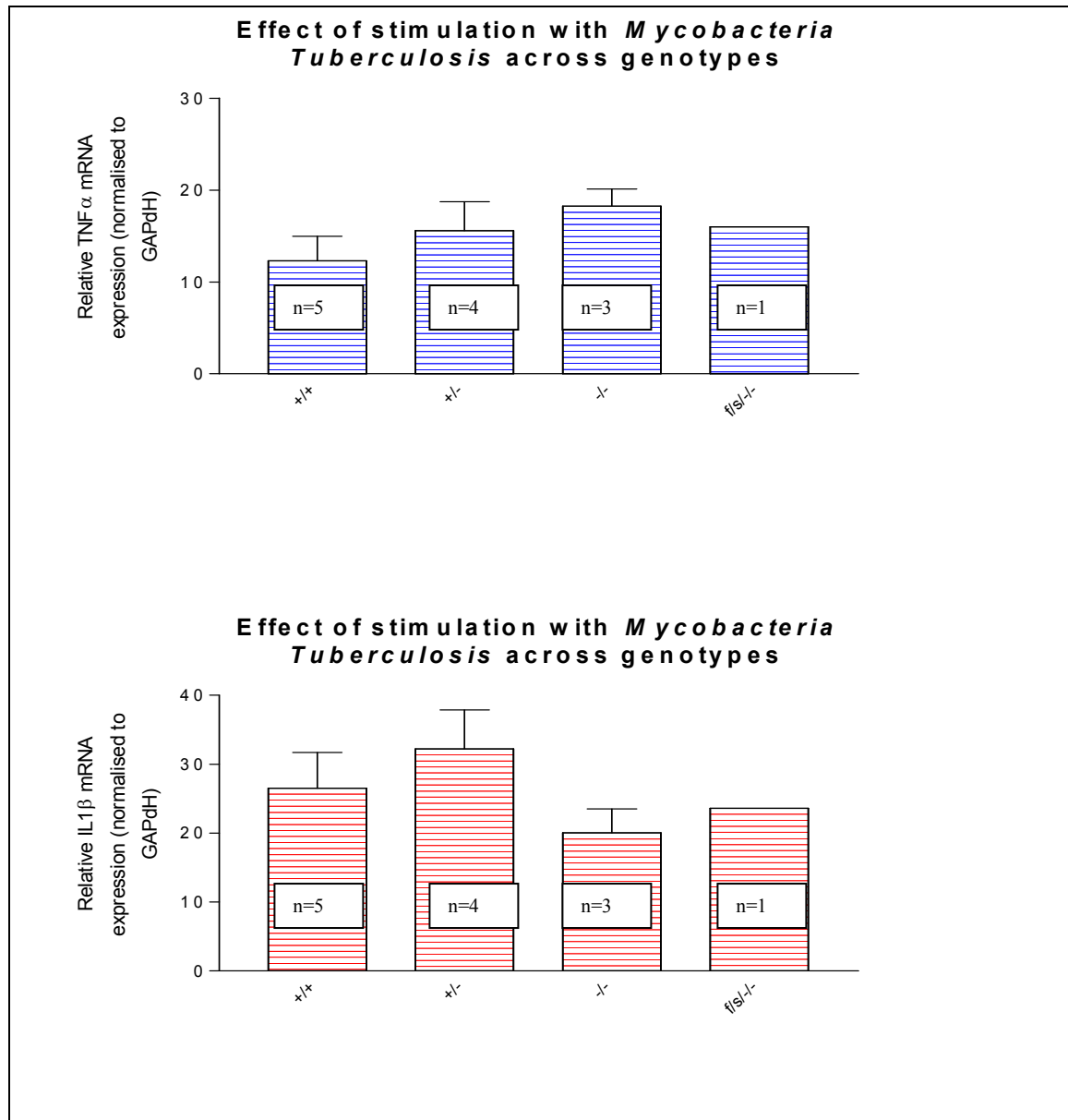


Figure 3.17 – TNFα and IL-1β cytokine response to *Mycobacterium tuberculosis* stimulation by PBMC across NOD2 genotypes

The inflammatory response by PBMC to *Mycobacterium tuberculosis* was more potent than the response to MDP but again less pronounced than the response to LPS. Firstly the cytokine response in PBMC from Crohn's patient's wild type for the NOD2 mutations was similar to that seen in healthy controls (data not shown.). *Mycobacterium tuberculosis* induced a mean 15.1 fold increase in TNFα expression and 26.8 fold increase in IL-1β

expression in PBMC from Crohn's patients. Furthermore there was no significant difference between the Crohn's patients regardless of their NOD2 status.

3.7.3 Effect of *Mycobacterium Vaccae* on PBMC across NOD2 genotypes

The effect of *Mycobacterium vaccae* on PBMC can be seen in Figure 3.18. *Mycobacterium vaccae* is also a modest inducer of pro inflammatory cytokines causing a mean 11.7 fold induction in TNF α expression and 20.2 fold induction in IL-1 β expression in PBMC. Again there was no significant difference across the patients carrying one or two disease causing NOD2 mutations or when specifically looking at PBMC with double frameshift mutation.

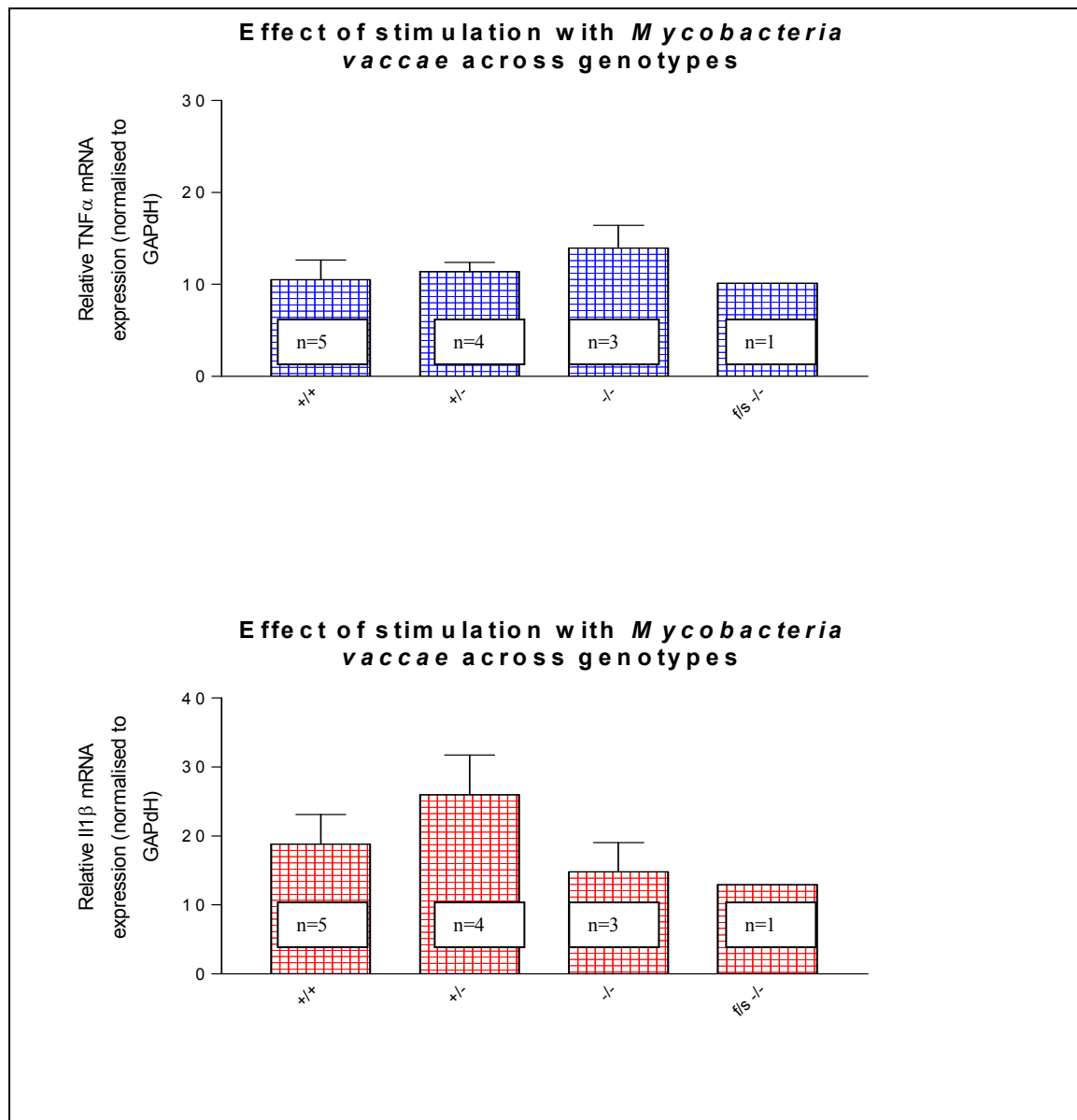


Figure 3.18 – TNF α and IL-1 β cytokine response to *Mycobacteria vaccae* stimulation by PBMC across NOD2 genotypes

3.8 Conclusion

I have demonstrated that PBMC from healthy controls respond to bacteria in a predictable stimulatory hierarchy when stimulated with MDP, PGN and LPS. LPS is the most potent stimulator where it causes a 54 fold increase in TNF α expression and a 59 fold increase in IL-1 β expression. No effect on the antiinflammatory cytokine TGF β expression was demonstrated.

PBMC from patients with Crohn's Disease also responded in a similar pattern to bacterial stimulation with LPS being the most potent inducer of inflammatory cytokines and MDP being the most modest. The effect of LPS on PBMC is unaffected whether you have no, one or two disease causing NOD2 mutations. This is for the reason that LPS is sensed through the surface receptor TLR4.

MDP is a modest stimulator of PBMC where it causes a mean 3.11 fold induction in TNF α expression in healthy controls and 2.64 fold induction in patients with Crohn's disease. In patients carrying two NOD2 mutations the stimulatory effect is reduced further particularly in PBMC homozygote for the frameshift mutation. This confirms the findings of the transfection studies (Inohara 2003, Girardin 2003) and also of Li (2004) who subsequently published their comprehensive studies of primary mononuclear cells in CD patients comparing the functional effects of the individual disease-associated mutations. They too demonstrated a decreased transcriptional response to MDP of the frameshift mutation.

Furthermore this data does not support the hypothesis that *Mycobacteria* depend on NOD2 for sensing. The stimulatory response of freshly extracted PBMC to *Mycobacterium vaccae* and *Mycobacterium tuberculosis* was modest when examining TNF α and IL-1 β cytokine expression and was not dependent on NOD2 status. This is in apparent contrast with the results of Ferweda (2005) and colleagues who demonstrated decreased TNF α protein in PBMC expressing mutant NOD2 proteins (encoded by the frameshift mutation) which were stimulated by sonicated MTB preparations. Possible reasons for the differences will be discussed in Chapter 4

Chapter Four

4.0 Determining the effect of MDP priming on PBMC response to bacterial stimulation.

4.1 Introduction

Muramyl dipeptide is a weak agonist inducing TNF α and IL1 β expression in PBMC. In PBMC expressing mutant NOD2 proteins, this response is further reduced: this is particularly so in PBMC expressing mutant NOD2 proteins encoded by the L3020insC frameshift mutation in the NOD2 gene (Inohara 2003, Li 2004). In the previous chapter (Chapter 3), I have shown similar results in freshly extracted human peripheral blood monocytes. Nonetheless, these results present an apparent paradox as Crohn's Disease is characterized by increased NF κ B activity and Th1 cytokine responses that are characterized by elevated levels of TNF α and IL1 β .

In mice, the combination of MDP and LPS induces greater inflammatory responses than either component used alone. Simultaneous intravenous injection of LPS and MDP results in marked lethality in guinea pigs and mice (Ribi 1979, Takada 1987). Ribi et al first observed that the administration of intravenous LPS and MDP induced early shock, which was sometimes lethal, in guinea pigs; animals receiving either LPS or MDP alone did not have such a reaction (Ribi 1979)

Similarly, Takada and colleagues showed that simultaneous administration of MDP and LPS was lethal to mice whereas administration of either MDP or LPS alone was not. They then went on to examine the optimal time interval between MDP and LPS injection required to sensitize mice for lethal toxicity of LPS. Takada demonstrated that pretreatment of 4 hours with MDP was more effective than either simultaneous administration or 2 hours pretreatment and after 4 hours pretreatment with MDP, LPS also caused an early anaphylactoid reaction which was absent in the other time periods. For this reason an interval of 4 hours between the MDP treatment and subsequent LPS treatment was observed in my experiments. (Takada 1987)

Furthermore the same group has shown that MDP priming is also effective for inducing increased inflammatory cytokines in mice in response to other bacterial components, including lipotechoic acid (LTA). (Monodane 1997)

MDP also appears to prime inflammatory responses in human monocytic cells. Yang and colleagues studied the effects of MDP in human monocytic cell lines THP-1 and U937 and demonstrated that MDP by itself exhibited only a weak ability to induce secretion of inflammatory cytokines such as IL-8 but showed marked synergistic effect with both LPS and LTA. They also found that cells primed with MDP exhibited enhanced production of IL-8 upon stimulation with LPS, while the cells primed with LPS showed no change in production upon stimulation with MDP. (Yang 2001)

In 2004, Uehara and colleagues showed that NOD2 is responsible for the synergistic effects of MDP and TLR agonists in monocytic cell lines. Their results suggested that combinatory dual signaling through extracellular TLR's and intracellular NODs might lead to the synergistic activation of host innate immune responses to invading bacteria. (Uehara 2004)

Having recognized this phenomenon in both animal models and monocytic cell lines I wanted to determine whether MDP modulated immune responses to bacterial antigens in human PBMC expressing wild-type and mutant NOD2 proteins. I hypothesized that MDP by itself is a weak agonist of inflammatory responses in monocytes but that MDP priming enhances subsequent inflammatory responses to bacterial ligands. I chose to 'prime' or pre treat the PBMC for 4 hours before subsequent LPS treatment on the basis of Takada's experiments which showed that 4 hours pretreatment with MDP is more effective than either simultaneous administration or 2 hours pretreatment. (Takada 1987)

4.2 MDP Priming of PBMC in healthy controls

Peripheral blood monocytes from twelve healthy controls were freshly isolated and prepared (as previously described) at a cell concentration of 4×10^6 cells/well in serum free media. They were then incubated concurrently for 4 hours with or without MDP – priming – before being stimulated with LPS for a further 2 hours. The doses used were

- Muramyl dipeptide (20 μ g/ml)
- Lipopolysaccharide (LPS) (100ng/ml); these doses were unchanged

All cells were then harvested, RNA extracted and cDNA synthesized as previously described. Quantitative PCR looking at TNF α and IL1 β expression was performed and results normalised for GAPDH expression

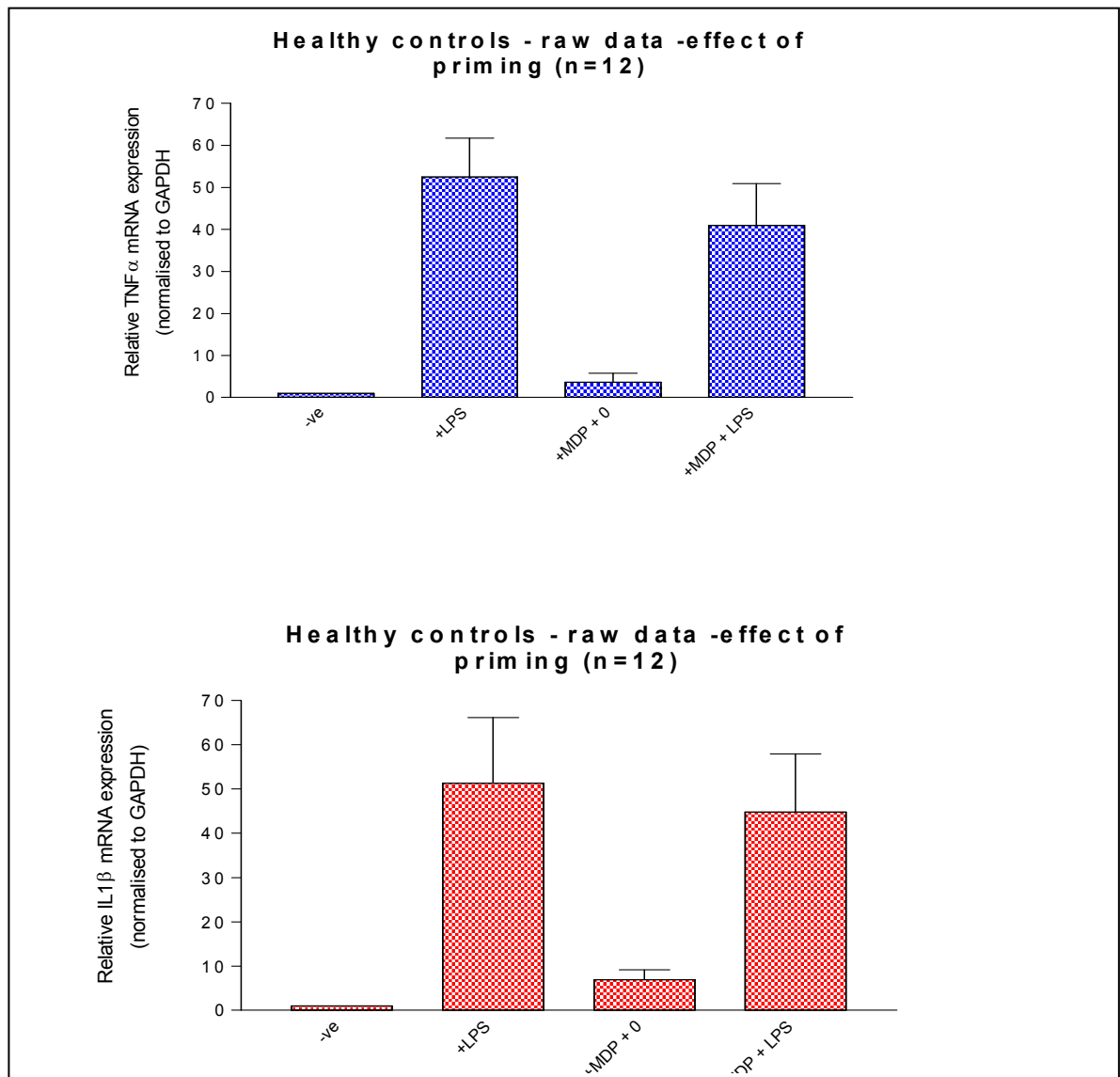


Figure 4.1-MDP modulation of TNF α and IL1 β mRNA expression in healthy PBMC

The effect of LPS on PBMC with or without priming with four hours of MDP is seen in Figure 4.1. The effect of LPS alone is consistent with that shown previously, causing a mean 51.7 fold induction in both TNF α and IL-1 β mRNA expression but, to our surprise cells primed for 4 hours with MDP had a reduced response to the subsequent LPS. LPS alone caused a mean 52.4 fold induction of TNF α expression but this was reduced to 40.9 fold if the cells had been primed with MDP and in terms of IL-1 β expression induction was reduced from a mean of 51.3 to 44.8 fold induction.

As expected using human PBMC there was considerable interpersonal variation so to allow us to compare results between patients I elected to set the effect of LPS alone at 100% and any changes are shown in relation to that. Figure 4.2 shows the same data re-analysed with the induction level caused by LPS alone set at 100%. We found that, priming with MDP significantly reduced the subsequent response of healthy monocytes to LPS from 100% to a mean of 77% in TNF α mRNA expression. This is statistically significant ($p < 0.05$). IL-1 β expression was also significantly down regulated after MDP priming from 100% to 82.4% ($p < 0.05$).

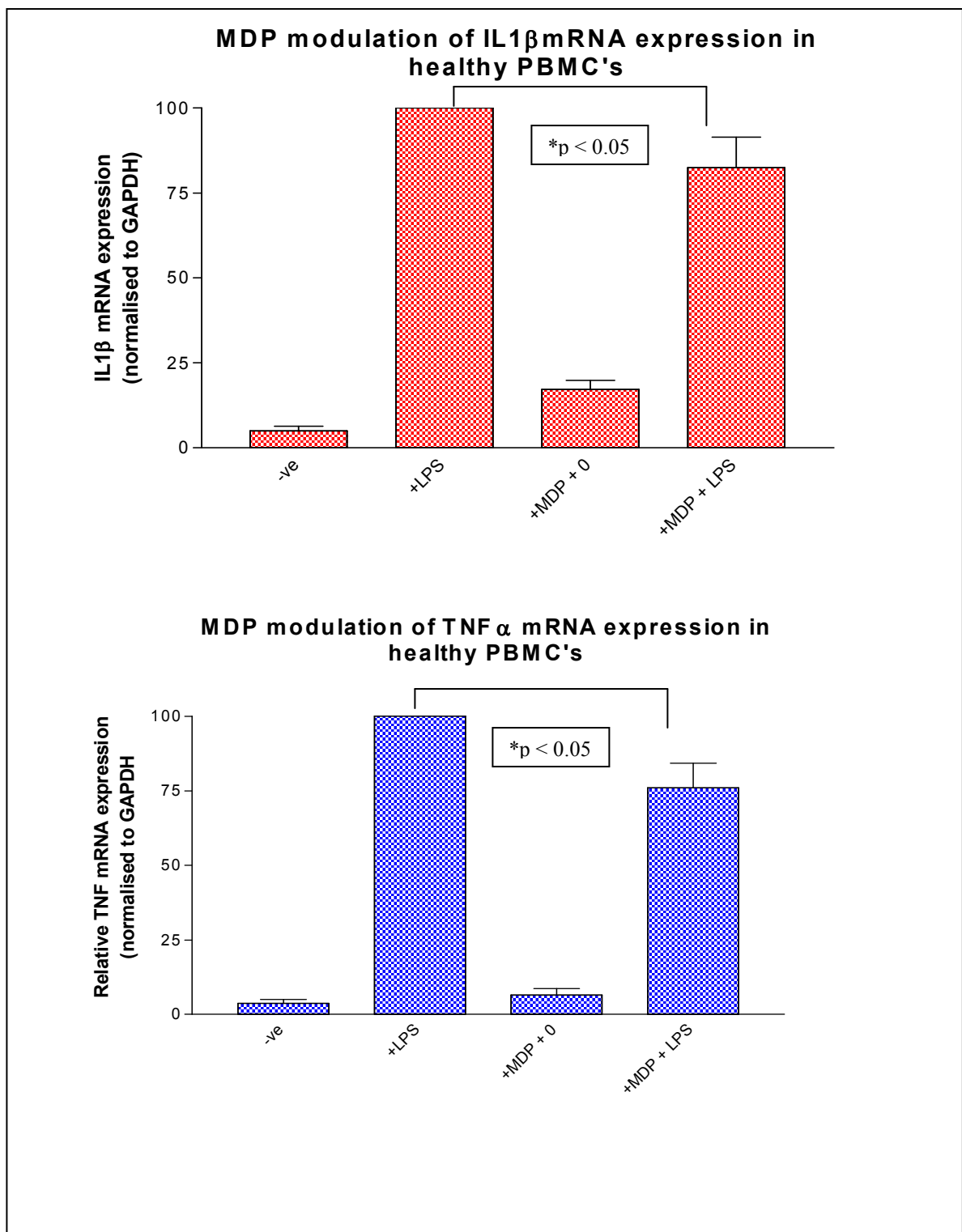


Figure 4.2-MDP modulation of TNF α and IL1 β in mRNA expression in healthy PBMC (n=12)

4.2.1 PGN Priming of PBMC in healthy controls

Prior to this and as part of my pilot studies I also however examined the effect of PGN 'priming' on monocytes subsequent response to LPS. I used the same experimental technique. I hypothesised that PGN would have its effect on monocytes via the breakdown product muropeptides and this may be sensed by NOD2. After performing the experiment on 5 healthy controls the 'priming' effect was seen with PGN but was more pronounced with MDP and thereafter I concentrated on MDP and used this in all NOD2 mutants.

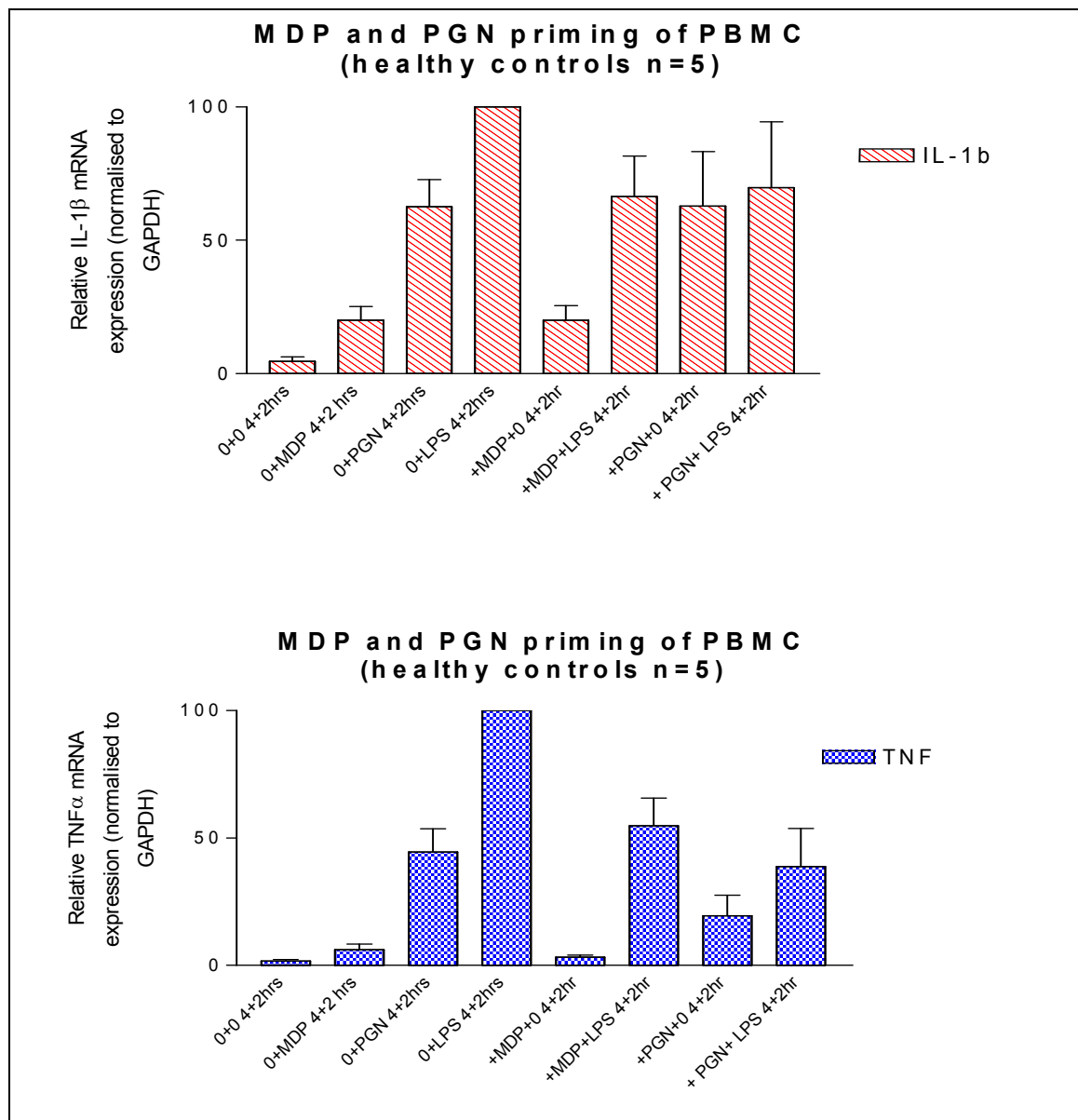


Figure 4.3 - MDP & PGN modulation of TNF α and IL1 β in mRNA expression in healthy PBMC (n=5)

4.3 MDP Priming of PBMC from Patients with Crohn's Disease

I then went on to examine the effect of MDP priming on PBMC from patients with Crohn's disease who were wild type, heterozygous or homozygous for the common disease causing mutations in the NOD2 gene. Freshly extracted PBMC from twenty-eight patients with Crohn's disease who attend the Inflammatory Bowel Disease clinic at the Royal Free Hospital, London were examined to determine whether the same phenomenon occurred. The clinical characteristics of the patients with Crohn's disease examined are previously described in Tables 3.1-3.3.

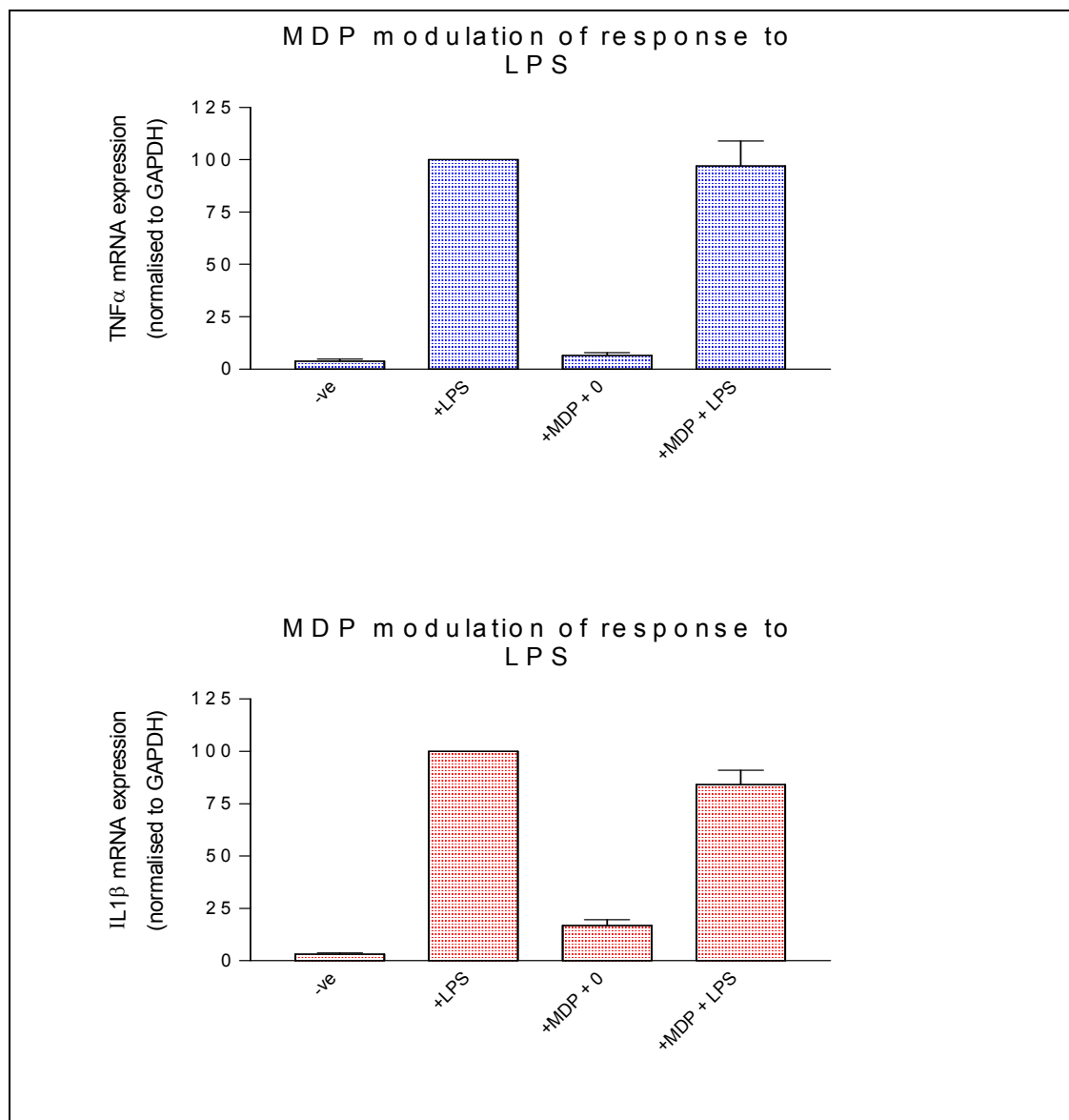


Figure 4.4 - MDP modulation of TNF α and IL-1 β mRNA expression in PBMC from Crohn's patients (n=28)

The same protocol as described in section 4.2 was followed with freshly extracted PBMC being stimulated with LPS for 2 hours after 4 hours priming either with or without MDP. All cells were then harvested, RNA extracted and cDNA synthesized as previously described. Quantitative PCR was performed looking at TNF α and IL-1 β expression. The response to LPS alone was set at 100% and any changes are shown in relation to that. The results are seen in Figure 4.4.

Whereas in healthy PBMC, 4 hours MDP pretreatment reduced the subsequent response to LPS from 100% to 77%, in PBMC from patients with Crohn's disease MDP pretreatment caused minimal change to the subsequent response to LPS -100% to 97% in terms of TNF α expression and 100% to 85% when measuring IL-1 β expression. It therefore appeared that MDP did not have the same down regulatory affect previously demonstrated in healthy PBMC in PBMC from patients with Crohn's disease.

As MDP is the minimal structural motif recognized by NOD2 we therefore speculated whether this priming effect may be modified by carrying mutations in the NOD2 gene and whether this was the explanation of the differing results between PBMC from healthy controls and Crohn's patients. We therefore looked at the effect of MDP priming on Crohn's patients separately who were wild type, heterozygous or homozygous for the common disease causing mutations in the NOD2 gene. There are as before 12 wild type Crohn's patients, 11 Heterozygote and 5 homozygote or compound heterozygote Crohn's patients.

4.4 MDP Priming of PBMC across NOD2 genotypes

4.4.1 MDP Priming of NOD2 +/+ PBMC

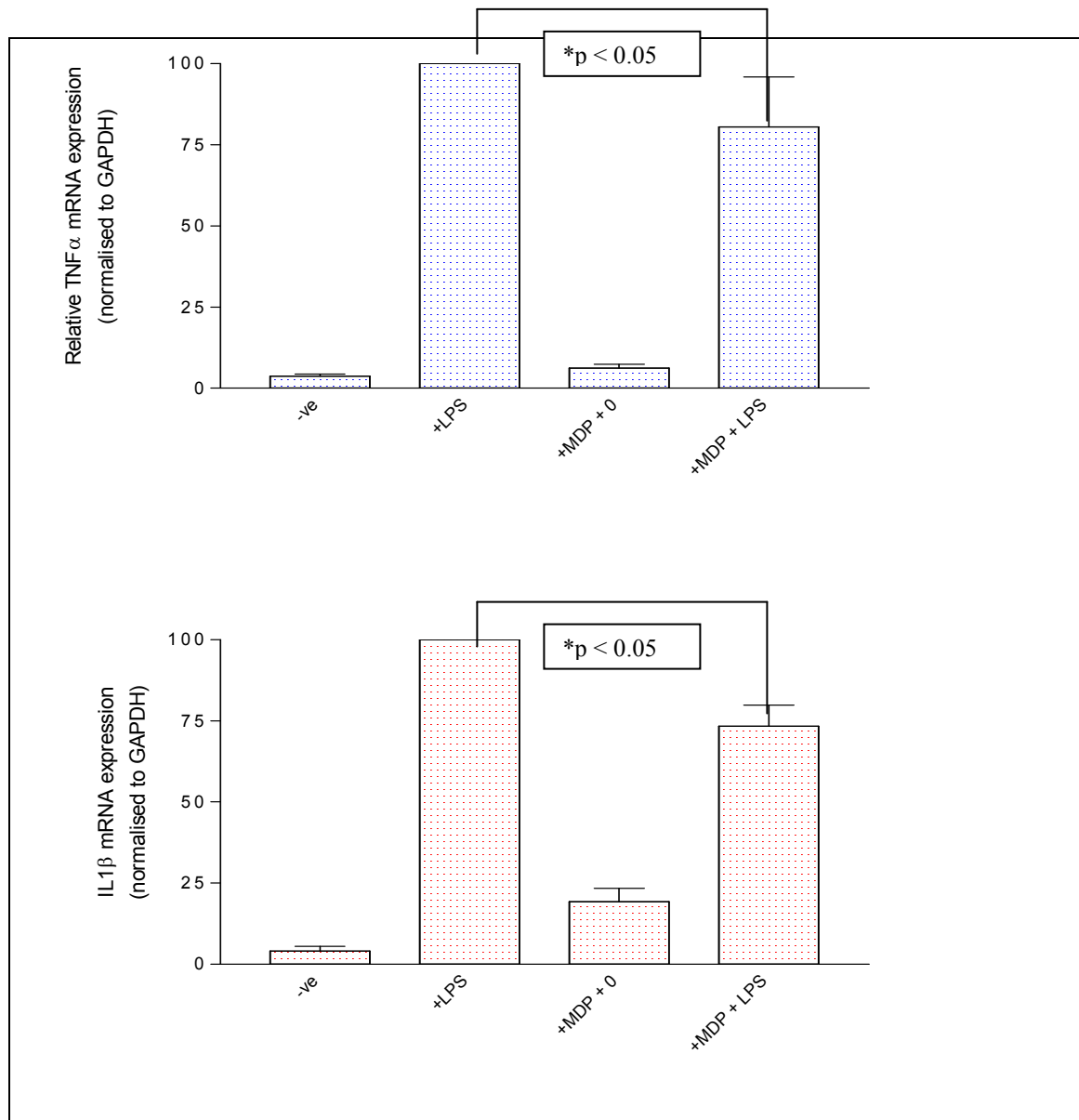


Figure 4.5-MDP modulation of TNFα and IL1β in mRNA expression in PBMC from NOD2 +/+ Crohn's patients (n=12)

Figure 4.5 demonstrates that in patients' wild type for the common disease causing NOD2 mutations MDP priming does cause a subsequent significantly reduced response to LPS stimulation similar to that seen in PBMC from healthy controls. MDP priming caused a reduction in TNFα expression from 100% to 76% and IL-1β mRNA expression was reduced from 100% to 72%. These are both statistically significant.

4.4.2 MDP Priming of NOD2 +/- PBMC

Examining patients who are heterozygous for NOD2 mutations the down regulatory modulatory response caused by 4 hours pretreatment with MDP is still present. The effect of LPS is reduced from 100% to 79% after MDP priming when measuring TNF α expression and from 100% to 75.7% when measuring IL-1 β mRNA expression.

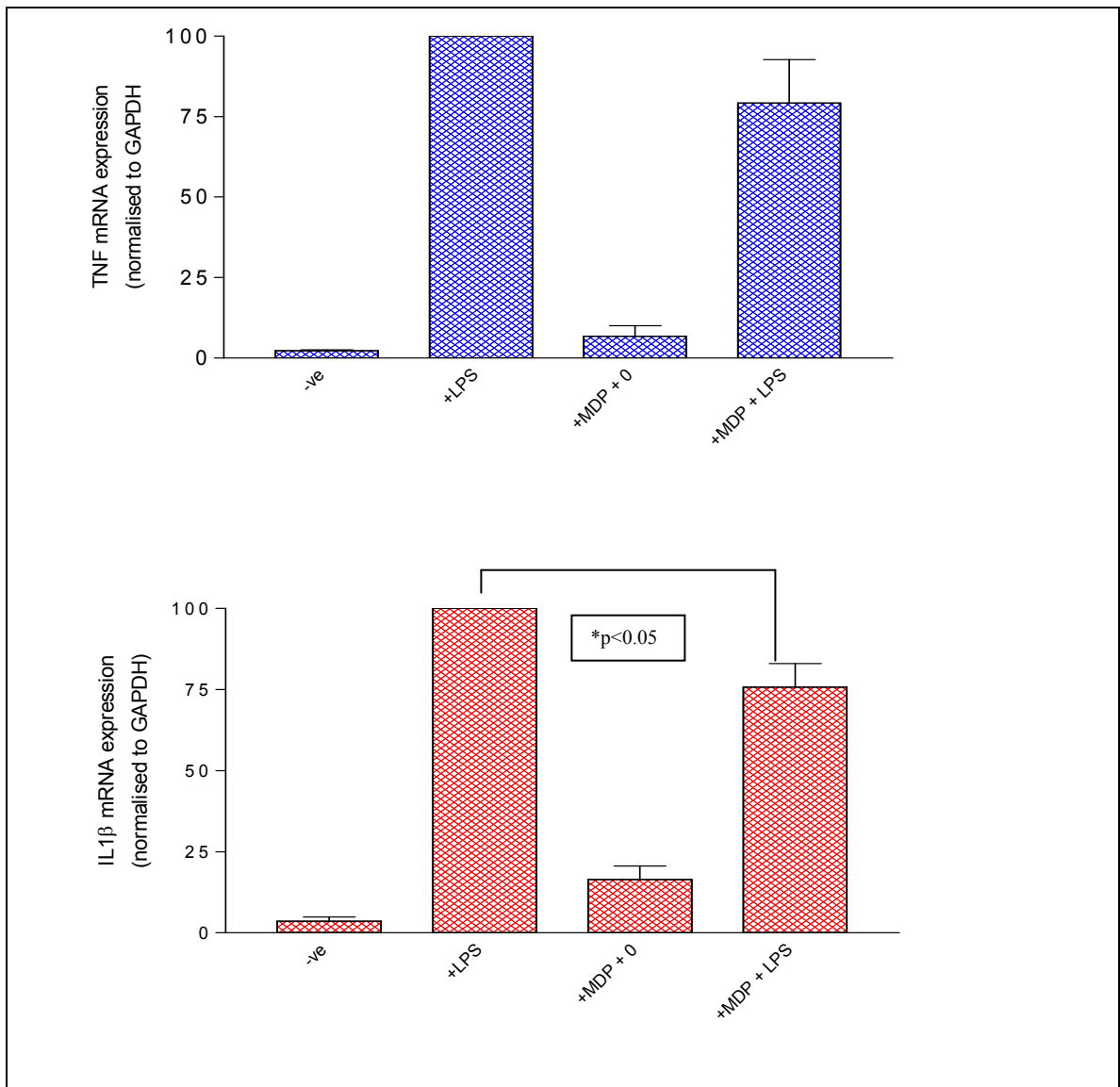


Figure 4.6-MDP modulation of TNF α and IL-1 β in mRNA expression in PBMC from NOD2 +/- Crohn's patients (n=11)

4.4.3. MDP Priming of NOD2 ^{-/-} PBMC

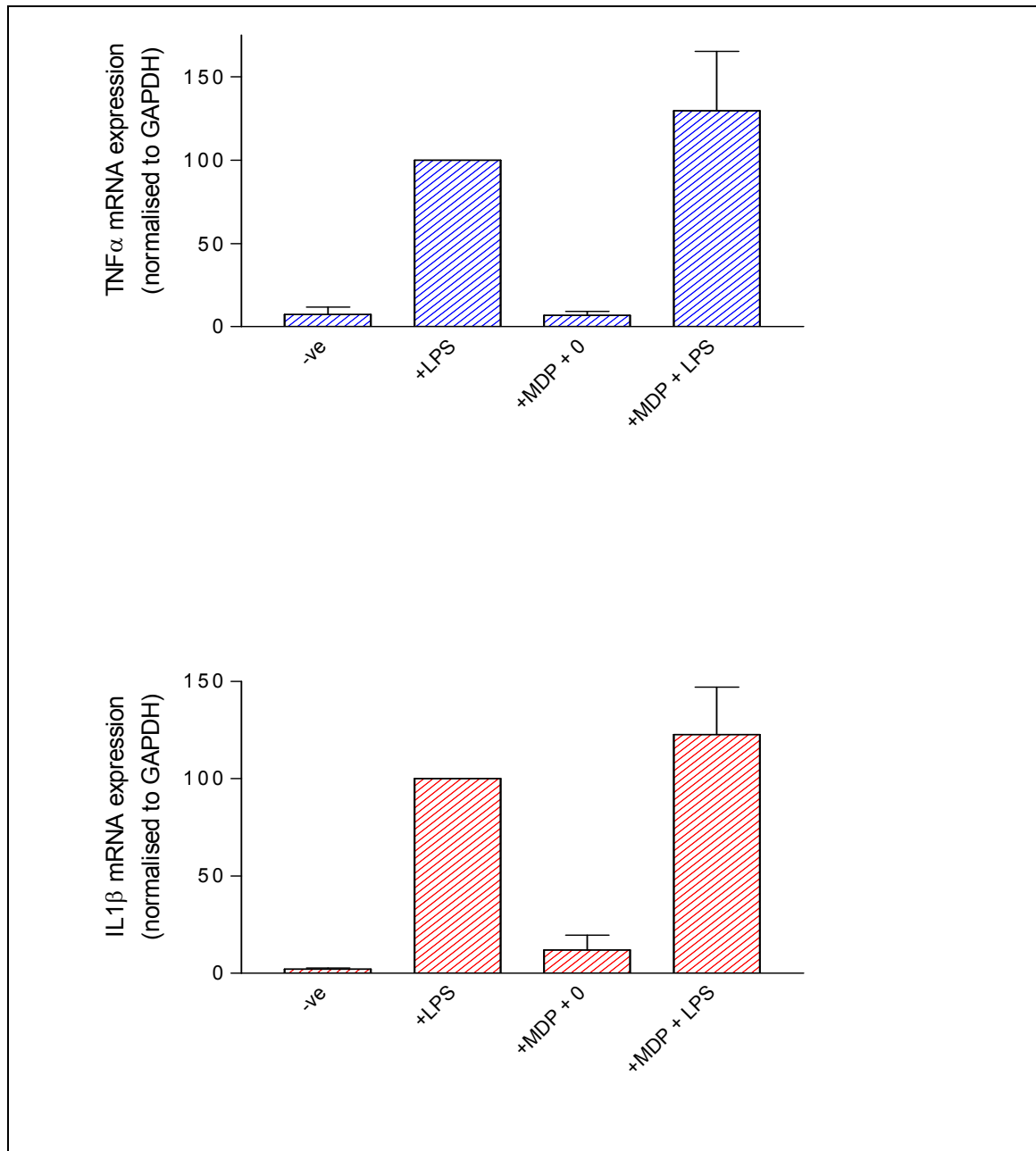


Figure 4.7-MDP modulation of TNF α and IL1 β in mRNA expression in PBMC from NOD2 ^{-/-} Crohn's patients (n=5)

When examining patients carrying two disease causing mutations in the NOD2 gene the down regulatory effect caused by MDP priming is lost and indeed the response to subsequent LPS is actually increased. TNF α mRNA expression was increased from 100% to a mean of 129.5% and IL-1 β from 100% to 122.8%. It should be emphasised that this effect observed is greater than the additive effects of MDP and LPS.

4.4.4 MDP Priming compared across genotypes

Whilst this apparent reversal of effect in patients who were homozygous is exciting the data must be taken with caution as when we look at the same data as individuals on the scatter graph in Figure 4.8 there is significant inter individual variation although the trend is still clear.

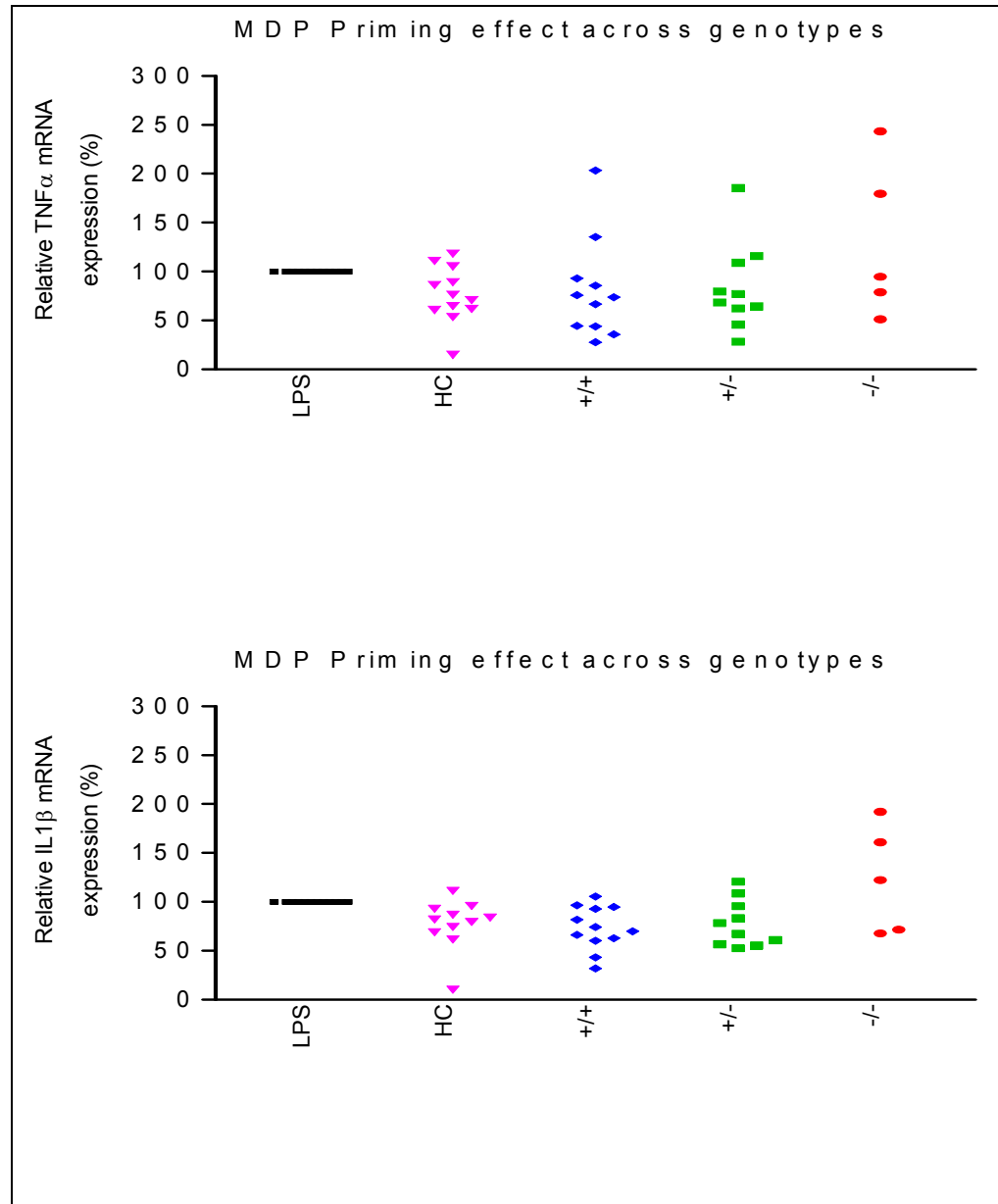


Figure 4.8-MDP modulation of TNF α and IL-1 β in mRNA expression in PBMC from across NOD2 genotypes

4.4.5 Summary

In PBMC from healthy controls and patients' wild type for the common disease causing NOD2 mutations, MDP priming for four hours causes a subsequent significantly reduced response to LPS and TLR4 mediated immune stimulation in terms of TNF α and IL-1 β mRNA expression. In Crohn's disease patients homozygous for NOD2 mutations this down regulatory effect of MDP stimulation of NOD2 is lost and indeed the subsequent response to LPS is increased. It will be important to confirm these findings in the future in a larger patient cohort who are homozygous both in the frameshift mutation and point mutations.

4.5 Discussion

4.5.1 Human Studies

Since I started my research a synergistic response between NOD2 stimulation by MDP and various TLRs has been published both in human and mouse models. These results appear to contrast with my own, and I will discuss possible reasons that may account for these differences. Whilst all confirm that MDP modulates the response to TLR stimulation, some show decreased cellular activation (i.e. loss of function) whilst others show increased cellular activation (i.e. gain of function). I will discuss the mouse models later (Chapter 6) but to compare my results with the published human findings (van Heel 2005 & 2005a) I will first discuss the differences in experimental technique here between them

The first report of synergistic enhancement of LPS responses by MDP, the NOD2 ligand was reported in monocytes by Traub. (Traub 2004) They initially hypothesised that this was due to cross talk between extracellular TLR signalling pathways and intracellular NOD2.

Following this van Heel and colleagues published two papers in 2005 analysing firstly, the cytokine response of PBMC to varying doses of MDP and secondly the effect of co-incubating MDP with different toll like receptor ligands (van Heel 2005). Using cryopreserved cells from seven healthy controls wild type for NOD2 and nineteen NOD2 double mutant genotypes, PBMC were incubated for 22 hours with MDP and either TLR 2,4,5 or 6 ligands before analyzing cytokine protein secretion using ELISA-based

techniques. They showed that MDP alone induced little TNF α or IL-1 β protein secretion (3.9% and 2.3% of amount induced by LPS) but strong IL-8 protein secretion after 22 hours incubation (33.6% of the response to LPS). They also confirmed previous findings that the response to MDP is reduced in NOD2 double mutants. This was particularly evident at low MDP concentrations and in the frameshift mutations. Van Heel et al then demonstrated that co-administration of MDP and each of the TLR ligands significantly increased TNF α and IL-1 β protein secretion. This upregulatory effect was abolished in cells carrying two NOD2 disease causing mutations

In their second paper van Heel and colleagues specifically examined the effect of the interaction of TLR9 ligand (CpG DNA) with MDP (van Heel 2005a). Again they used cryopreserved PBMC and examined protein cytokine expression by ELISA from 7 healthy controls and 19 Crohn's patients who carried the NOD2 double mutant genotype. They showed that concomitant MDP and TLR 9 stimulation caused a marked increase in TNF α (2.1 fold) and IL-8 (3.7 fold) protein secretion. This effect was also abolished in the double NOD2 mutant genotype.

Van Heel concluded that NOD2 activation by MDP causes an amplification of the innate immune response to other pathogens via the TLR system and proposed that the absence of this in NOD2 associated Crohn's disease causes failure of the early pathogen clearance and thus an abnormal adaptive immune response.

4.5.2 Possible Confounding factors

My data show that MDP priming modulates cellular response to LPS, and that this effect is likely to be mediated by the NOD2 protein: MDP-primed PBMC expressing the mutant NOD2 proteins encoded by a homozygous mutation in the NOD2 gene increase mRNA transcription of TNF α and IL-1 β after LPS stimulation. Although I did not measure protein secretion in my experiments (due to financial and time constraints) my results contrast with those reported by van Heel and colleagues.(van Heel 2005 & 2005a) It is therefore imperative to exclude possible confounding factors, examine differences and to ensure that the only predictive difference is in the patients NOD2 status. I will therefore discuss the differences and possible confounding factors in my study here.

4.5.3 MDP concentrations

The concentration of MDP which is relevant both in bacterial infection and bacterial health in humans is not known and varying doses have been used. In 2004 Traub demonstrated synergy between muropeptides and LPS in human whole blood both at the translational and transcriptional level. This was particularly evident at nanomolar concentrations of MDP where they showed that as little as 10ng/ml MDP increased the TNF α releasing capacity of LPS 4-fold. They also examined IL-1 β , IL-6 and IL-10 and showed the effect was consistent though less pronounced in these other cytokines.

Li and colleagues (Li 2004) in their paper showed varying responses to MDP across genotypes and doses. In wild type individuals, MDP-induced IL-8 protein expression with a greater response to high dose (1mcg/ml) compared with low dose (10ng/ml) MDP. At low MDP doses, in all homozygotes they observed no induction of IL-8 protein. With higher doses of MDP only the Leu1007fsins C mutation did not respond.

Van Heel in their studies demonstrated that the differences between homozygote cells and wild type cells in terms of IL-8 secretion after stimulation with MDP alone was particularly pronounced at low nano molar concentrations of MDP (10ng/ml) but the difference was still present though smaller, at higher concentrations. This suggests a change in sensitivity rather than an all or nothing effect. Thereafter van Heel used MDP at a dose of 10ng/ml as the maximal difference between wild type and double mutant cells was seen at this concentration and went on to show that MDP at this dose substantially up regulated secretion of TNF α and IL-1 β induced by TLR ligands and that this effect was abolished in cells carrying two NOD2 disease causing mutations.

MDP because of its low molecular weight and water solubility is rapidly excreted from the body. After intravenous injection of MDP at 1.5mg/kg into rats, plasma levels immediately start to decline (Fosset 2003) such as at thirty minutes after injection the plasma levels fall from 27 μ g/ml to 4 μ g/ml and MDP was no longer detectable after 2 hours.

In summary the concentrations of MDP which may be relevant both in bacterial infection and bacterial health in humans is not known and how these varying doses affect cellular responses is not clarified. The concentrations of MDP we used were considerably higher

compared to van Heel and colleagues and may be a factor to explain the difference between the results.

4.5.4 Clinical Aspects

As stated previously my results differ from other published human data on the modulatory effect of MDP on PBMC responses and another possible difference are the clinical aspects of the patients studied.

4.5.4.1 Presence of Disease

Van Heel compared healthy controls, with normal NOD2 with Crohn's disease patients with double NOD2 mutants. They did not examine patients with Crohn's with normal NOD2 function so theoretically the differing result between the two groups may be due to the presence of Crohn's disease itself and the NOD2 status is irrelevant. This is unlikely however as they report that the PBMC from both groups had similar responses to LPS alone (unpublished data)

Li et al in their study (Li 2004) of the effect of MDP mediated activation of PBMC demonstrated varying sensitivity of the Crohn's disease associated NOD2 mutations in their response to MDP. However they also demonstrated that the absolute levels of IL-8 secretion were significantly higher in wild type Crohn's individuals compared to wild type healthy controls suggesting the presence of the disease is a factor in how PBMC respond to MDP.

4.5.4.2 Disease Activity

It is also important to exclude whether the presence of active inflammation may be a confounding factor in our results particularly as van Heel in his papers had compared healthy controls against Crohn's disease patients with double NOD2 mutants and stated that the patients had predominantly quiescent disease. In our cohort it is noted that there were many patients with active disease at the time of venesection particularly in the NOD2

homozygote cohort as compared to either the wild type or heterozygote groups. 60% (3/5) of the patients carrying 2 NOD2 mutants had active disease compared with only 2 of the 12 (16.7%) patients wild type for the common disease causing NOD2 mutations and 4 of the 12 heterozygote (33%) for the common NOD2 mutations. However when examining the results of these individual patients in my experiment the presence or not of active disease did not predict the response to MDP priming. It remains possible however that the differences may be due in part to the confounding effect of inflammation. One possible explanation for this discrepancy is that after prolonged exposure to high levels of proinflammatory cytokines and bacterial products in the circulation, these monocytes may have become refractory to further stimulation.

4.5.4.3 Treatment

Another possible explanation of the differences is the confounding effect of medical treatments particularly immunosuppressant therapy. Amongst my cohort immunosuppressant use with thiopurines or methotrexate was proportionally higher amongst patients' wild type for NOD2 mutations. 58.3% of patient's wild type for the common NOD2 mutations were on immunosuppressive treatment at the time of venesection compared to 40% of patient's homozygote for NOD2 mutations and 33% of heterozygote patients but again looking at individuals their use did not predict response to MDP priming. Van Heel commented that there was no difference between the response in patients on or off immunosuppressive treatment (van Heel 2005).

There were only one current user of Infliximab and they were in the homozygote group. There were 2 from each of the heterozygote and wild type groups who had previously been exposed to Infliximab and again despite inter individual variation this did not enable us to predict the response.

4.5.5 Transcriptional / translational level

Traub (Traub 2004) demonstrated synergy between LPS and MDP at both the translational and transcriptional level in PBMC. Wolfert (Wolfert 2002) also studied the effect of synergy examining it in the human monocytic cell line MonoMac 6 cells. Interestingly they showed that stimulating MonoMac 6 cells with MDP alone induced a 10 fold induction of TNF α mRNA but this did not result in increased TNF α protein expression. Therefore the majority of TNF α mRNA induced by MDP is not translated into protein. They suggest that MDP induces TNF α gene expression specifically without TNF α translation. They went on to demonstrate synergy in the MonoMac 6 cells and reported an increased transcription of LPS induced TNF α mRNA when MDP was co-incubated with LPS. They suggest that the apparent synergy between LPS and MDP is due to the removal of the block in translation in the presence of LPS.

Li (Li 2004) and colleagues as stated previously demonstrated that MDP induces a broad array of transcripts including IL-1 β , TNF α and IL-8 and that in PBMC with double Leu1007fsinsC mutation there is reduced response to MDP. Furthermore they demonstrated that in Leu1007fsinsC homozygotes there was a profound defect in IL-1 β protein secretion despite a marked induction of IL-1 β mRNA after being stimulated with MDP and TNF α and suggested that this would indicate that the NOD2 pathway is involved in post transcriptional induction of IL-1 β expression

Van Heel et al in their studies demonstrated synergy in the cytokine response at the protein level after 22 hours of coincubation rather than examining the RNA level. Another key difference therefore is that we examined the mRNA level rather than the protein level. It is possible therefore that in our experiments TNF α and IL-1 β gene expression was specifically induced without translation into protein as Wolfert has previously suggested. (Wolfert 2002) It would be crucial and very interesting to examine the supernatants collected from my experiments to see if the induction and 'priming' effect is seen at the protein level.

4.5.6 NOD2 mutations

4.5.6.1 ? True recessive disease

We examined healthy controls and Crohn's disease-affected patient's wild type, heterozygote and homozygote for the common NOD2 mutations whereas van Heel in his study only compared healthy controls with 19 Crohn's patients that were homozygous for the NOD2 mutants. They elected not to examine patient's heterozygote for NOD2 mutations for two reasons. Firstly heterozygotes cannot be confidently identified due to the presence of rare variants which are known to comprise 24% of NOD2 homozygotes. (Lesage 2002) Secondly Li et al (2004) had reported no difference between wild type and heterozygous individuals in MDP stimulated PBMC IL-8 secretion and hypothesised that the genetic and biological data are consistent with an entirely recessive model of disease with no true heterozygote. They went onto suggest that heterozygous NOD2 carriers are actually misclassified double mutants carriers, carrying a second unidentified disease associated mutation.

In our cohort only the 3 common disease causing mutations Leu1007fsinsC, G908R and R702W have been identified. This may indeed underestimate the number of homozygotes and could have an effect on the results obtained in our heterozygote group.

4.5.6.2 Varying NOD2 mutations

In contrast to wild type and heterozygotes Li (Li 2004) showed there was an impaired response in the NOD2 double mutants or homozygotes to MDP which was most significant in the double dose Leu1007fsinsC mutation. In G908R and R702W homozygotes there was impairment of NF- κ B activity but this was more modest. Despite this varying MDP sensitivity they demonstrated in vivo each of the three risk alleles had comparable allele frequency and genetic risk. Van Heel however although showing that PBMC with NOD2 double mutants had impaired response to MDP compared to wild type PBMC, commented that there was no difference between the 5 different NOD2 double mutant types.

Further subdivision of the double mutants in our cohort into the Leu1007fsinsC frameshift or point mutations also did not predict how PBMC responded to MDP priming and

subsequent LPS stimulation. Of our cohort we have two patients homozygote for the Leu1007sinsC frameshift mutation, one homozygote for the R702W mutation and two compound heterozygotes but again this did not enable us to predict whether the response would be up or down regulatory in terms of MDP priming.

4.5.7 Blood

Peripheral blood mononuclear cells are heterogenous including B cells, T cells and monocytes. The synergistic effect could be dependent not only on a bystander effect from other blood cells but also from serum components. Traub and colleagues examined this and demonstrated that synergism was dependent purely on the monocytes (Traub 2004). Van Heel (2005a) and colleagues analysed the separate components by immunomagnetic beads in their study of peripheral blood mononuclear cells and found no significant difference between cell types.

A further difference is that we used freshly extracted PBMC whereas van Heel et al used cryopreserved cells. They state (van Heel 2005) that the cryopreservation was according to a method reported by Kreher to yield similar results in overnight cytokine assays to fresh cells (Kreher 2003). They confirmed this finding in their lab (unpublished). I performed their method of cryopreservation and repeated my experimental protocol with concurrent fresh and previously cryopreserved cells for 1 healthy control, 1 wild type and 1 homozygote and there was no difference in the pattern or trend but the absolute levels were lower in the cryopreserved cells. All other variables were kept constant. This factor therefore appears to be unlikely to explain the differing results but does suggest that cryopreservation of PBMC may be a useful strategy to perform future studies.

4.5.8 Technical Issues

Technical issues which were considered include the following. We were careful to use pure synthetic ligands (LPS, PGN and MDP) to avoid the risk of contamination by biologically active impurities and also eliminate variation in bacterial preparations

Another possible explanation is that the LPS was contaminated with MDP and so the effect we noted was due to the natural downward curve over time of stimulation with LPS but this is not the case as the effect of MDP alone was examined concurrently and had the expected minimal pro inflammatory effect.

Another clear difference is that there was concurrent stimulation of PBMC by van Heel's group versus 'priming' with MDP alone then subsequent stimulation in our experimental protocol. In vivo it is not known how innate immune pattern recognition receptors are triggered as there are many TLR and NOD ligand motifs present on a microbial pathogen.

4.6 Conclusion

In PBMC from healthy controls and patients' wild type for the common disease causing NOD2 mutations MDP priming for four hours causes a subsequent significantly reduced response to LPS stimulation in terms of TNF α and IL-1 β mRNA expression. In PBMC with double NOD2 mutant this down regulatory effect of MDP stimulation of NOD2 is lost and indeed the subsequent response to LPS is increased.

Van Heel has published other human data examining TLR and NOD2 stimulation in PBMC and they too show that MDP modulates the response to other pathogenic components via the toll-like receptor system, however whereas their data show a functioning NOD2 normally enhances the innate immune responses to TLR and this is lost in Crohn's disease patients homozygous for NOD2 mutations our data suggests the opposite. How this translates to the *in vivo* situation is as yet unclear as it is likely that innate immune pattern recognition receptors are not triggered one by one but rather that there is complex activation of multiple receptors taking place concurrently due to the many TLR and NOD ligand motifs present on a microbial pathogen.

Chapter Five

5.0 Stimulation of Intestinal Epithelial cells by bacterial ligands

5.1 Introduction

Mutations of the NOD2 protein are associated with increased susceptibility to Crohn's disease. The NOD2 protein is expressed in monocytes and Paneth cells and in previous chapters I have shown the results of work examining the response of monocytes carrying mutations in the NOD2 gene to bacterial products. With the more recent exciting discovery of NOD2 expression in Paneth cells and with emerging evidence that Paneth cell products may be regulated in NOD2 associated Crohn's disease (Wehkamp 2003) I would have liked to examine the effect of NOD2 mutations on Paneth cells function particularly in response to bacteria. Unfortunately this effect is more difficult to examine as Paneth cells from human tissues are difficult to obtain and there is no Paneth cell –like cell line.

Paneth cells express both the NOD2 protein and secrete lysozyme. I speculated therefore that Paneth cells may play another role in the development of intestinal inflammation that is mediated through the actions of the secreted antimicrobial product lysozyme. Muramyl dipeptide (MDP) is the minimally active component of peptidoglycan that stimulates NOD2-mediated cellular responses and it is a synthetic component. In biological systems, muropeptides (of which MDP is a commercially available example) are generated by the enzymatic action of lysozyme on the peptidoglycan component of the bacterial cell wall. Paneth cells are strategically located at the bases of the crypts of Lieberkuhn and secrete lysozyme in response to intestinal bacteria, an action that is likely to generate the production of muropeptides.

In this chapter I therefore examine the effect of lysozyme and lysozyme generated muropeptides on cellular responses in intestinal epithelial cells.

5.1.1 Lysozyme

Lysozyme (LZM) is an antibacterial enzyme originally described in 1922 by Alexander Fleming. Lysozyme is found at significant concentrations in many human secretions

including tears, breast milk, saliva and gastric and small intestinal secretions. (Elphick & Mahida, 2005) It is expressed in the intestinal tract by gastric and pyloric glands, duodenal Brunners glands, mononuclear cells and granulocytes but most strongly by Paneth cells. (Stamp 1992) Lysozyme is a 14kDa cationic enzyme which cleaves the glycosidic bonds that connects N-acetyl muramic acid with N-acetylglucosamine and which stabilises peptidoglycan, thereby resulting in bacterial lysis. Peptidoglycan (PGN) is the exoskeletal component of bacterial cell walls that provide bacteria with shape & mechanical rigidity. Lysozyme is predominantly active against Gram positive bacteria as Gram negative bacteria have an outer membrane which makes them relatively resistant to lysozyme. (See also Chapter 1.5.3.2.1)

5.1.2 Lysozyme as a Bactericidal Effector

Alexander Fleming also discovered and named a bacterial species now called *Micrococcus Luteus* which was highly susceptible to LZM mediated killing. He proposed that *M.luteus* and certain other bacteria are non pathogenic directly because they are readily destroyed by LZM. However lysozyme has also been reported to have a bactericidal activity independent of its enzymatic activity and its importance in innate immunity is suggested by the finding of reduced lysozyme protein staining in Paneth Cells in newborn infants with necrotising enterocolitis, a severe inflammatory intestinal disease that is often fatal. (Coutinho 1998) These authors suggest a deficiency of lysozyme, may render the intestine more susceptible to bacterial infection and bacterial translocation across the mucosa.

The role of LZM as a bactericidal effector has also been suggested by Akinbi and colleagues (Akinbi 2000) who studied mice transgenic for rat LZM. The increased production of lysozyme in the respiratory epithelial cells of the transgenic mice, compared to control mice enhanced bacterial killing in the lung *in vivo*, and was associated with decreased systemic dissemination of pathogen and increased survival following infection with Group B *Streptococcus* and *Pseudomonas aeruginosa*.

Furthermore Ganz and colleagues (Ganz 2003) examined transgenic mice deficient in LZM-M. Unlike humans who have a single LZM gene, mice have two, one encoding lysozyme M found in leukocytes and another encoding lysozyme P, normally expressed in

Paneth cells. After challenge with the normally non pathogenic and highly lysozyme-sensitive bacterium *Micrococcus luteus* they demonstrated more severe and extensive lesions in the lysozyme-M deficient mice than in the wild type mice. They proposed that the tissue injury was due to the failure of lysozyme-M deficient mice to inactivate PGN resulting in a prolonged and more severe inflammatory response and they proposed therefore that in healthy normal tissues, injury is normally limited by prompt degradation of the bacterial macromolecules that trigger innate immunity and inflammation.

Defects in innate immunity may contribute to the development of Crohn's disease, and as lysozyme has been implicated in innate immune responses (Ganz 2003) I wanted to examine the effects of lysozyme on inflammatory responses in intestinal epithelial cells.

5.2 Aims

The biologic function of lysozyme is not fully defined, an apparent paradox given its abundance and ubiquitous distribution in animal tissues. Paneth cells secrete lysozyme and it is very likely *in vivo* that lysozyme breaks down intestinal bacteria to generate muropeptides. To mimic this situation *in vitro* I used lysozyme to generate muropeptides and then used these to stimulate intestinal epithelial cells and thus explore the effect both lysozyme and lysozyme generated muropeptide stimulation has on inflammatory signaling in intestinal epithelial cells.

I furthermore wanted to determine whether lysozyme induced-inflammatory responses varied from different bacterial species as lysozyme may generate muropeptides that are specific to a particular bacterial species or strain.

Ideally, I would like to have examined these responses in Paneth cells but primary Paneth cells are difficult to obtain from human tissue and maintain in culture and there is no Paneth cell-like cell line. I therefore used an intestinal epithelial cell line SW480 as a surrogate. This cell line was chosen because our laboratory has shown that it has the highest level of NOD2 mRNA expression compared to other cell lines such as Caco-2 and HT29 cells, and also expresses human defensin 5 as measured by PCR. (Ogura 2003) All results obtained were analysed using students t- test.

5.3 Effect of LZM on SW480 responses to whole bacteria

I sought to determine the effect LZM had on the response of SW480 cells to bacterial stimuli. SW480 cells were propagated in T75 flasks and once 90% confluent were washed and transferred to 6 well plates in fresh culture medium and incubated for 16 hours at 37°C overnight. The SW480 cells were then stimulated with whole *M.luteus* 5 mg/ml either with or without LZM 100 mg/ml for 2-8 hours in a 37°C incubator.

The cells were then harvested at the end time point, RNA extracted and cDNA synthesized. qPCR was performed looking at gene expression. Results were normalized for GAPDH expression and an ethidium bromide gel was run to examine the size of the PCR products and to confirm the specificity of the reaction. The inflammatory cytokines examined initially were IL-8 and TNF α . Results shown are the results of at least triplicate experiments.

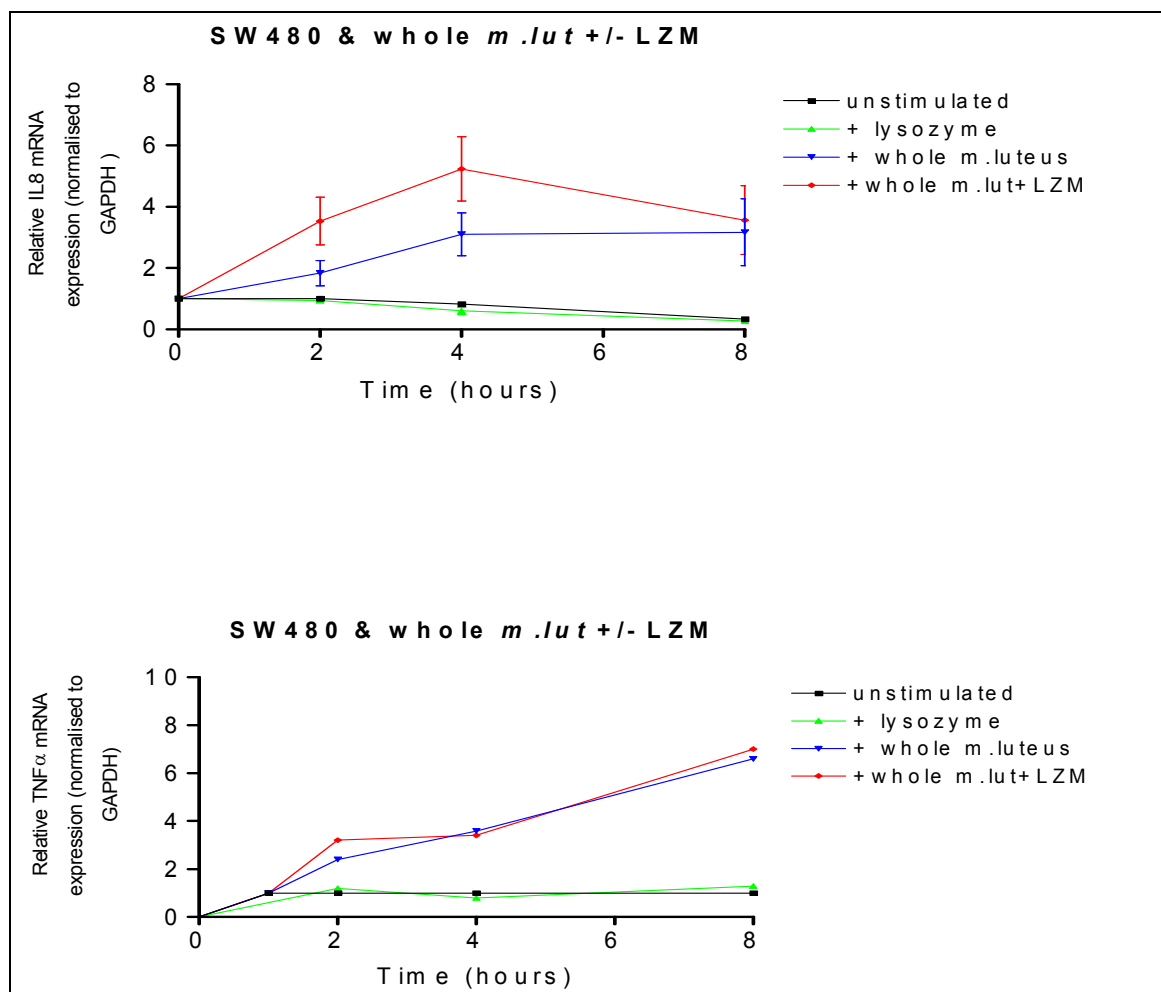


Figure 5.1 - Effect of *micrococcus luteus* +/- Lysozyme on IL-8 and TNF α mRNA expression in SW480 cells – Time course

The first result noted was that LZM alone has no effect on TNF α or IL-8 cytokine induction. The levels of these proinflammatory cytokines is the same as in the controls at all time points over an eight hour period. Whole *M.luteus* on SW80 cells induces IL-8 mRNA expression 2.5 fold after 4 hours before plateauing over the following 4 hours. The addition of LZM increases 2 fold the transcription of IL-8 mRNA but not TNF α at both 2 and 4 hours compared to stimulation with whole bacteria alone.

5.3.1 Effect of LZM on SW480 responses to whole bacteria – Dose response

Having established a time course and showing maximal difference at 4 hours I used the same method to establish a dose response effect. (Figure 5.2) This shows that the addition of LZM to *M.luteus* increases the transcription of IL-8 mRNA but not TNF α mRNA expression compared to stimulation with whole bacteria alone. The results also confirm that LZM alone has no effect on inducing IL-8 or TNF α mRNA expression in SW480 cells. *M.luteus* alone induces IL-8 mRNA and TNF α mRNA expression in increasing amounts relative to the dose of *M.luteus* but this proinflammatory effect was weak.

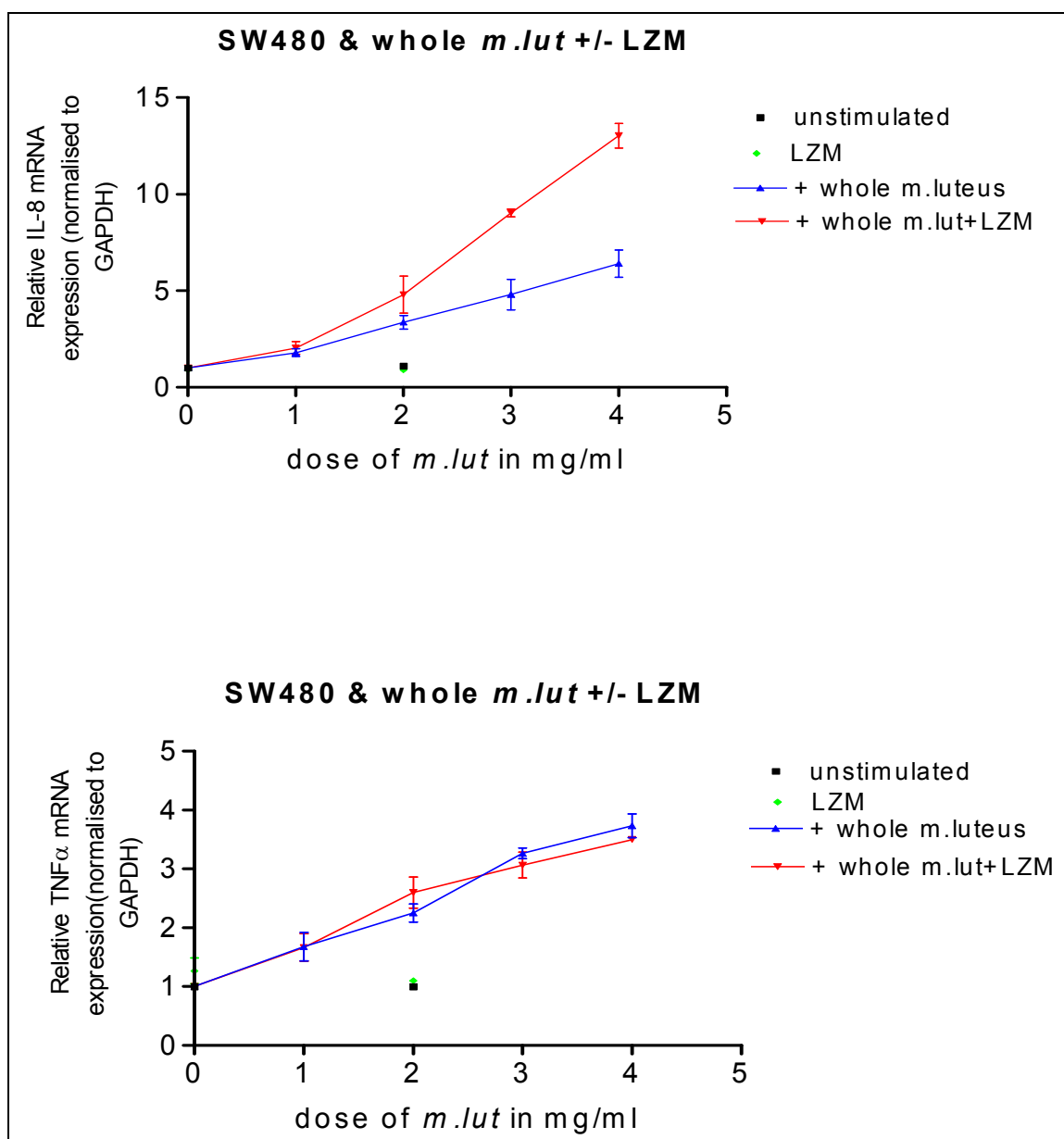


Figure 5.2 - Effect of micrococcus luteus +/- Lysozyme on IL-8 and TNF α mRNA expression in SW480 cells – Dose response

Based on these transcription mRNA results that lysozyme may affect IL-8 and other pro-inflammatory signaling cytokines I wanted to minimise the role of other possible bacterial antigens such as LPS and thus used commercially available PGN, the substrate on which lysozyme has its effect to determine the specificity of the cellular responses induced by lysozyme.

5.4 Effect of LZM on SW480 responses to PGN *Staph.aureus*

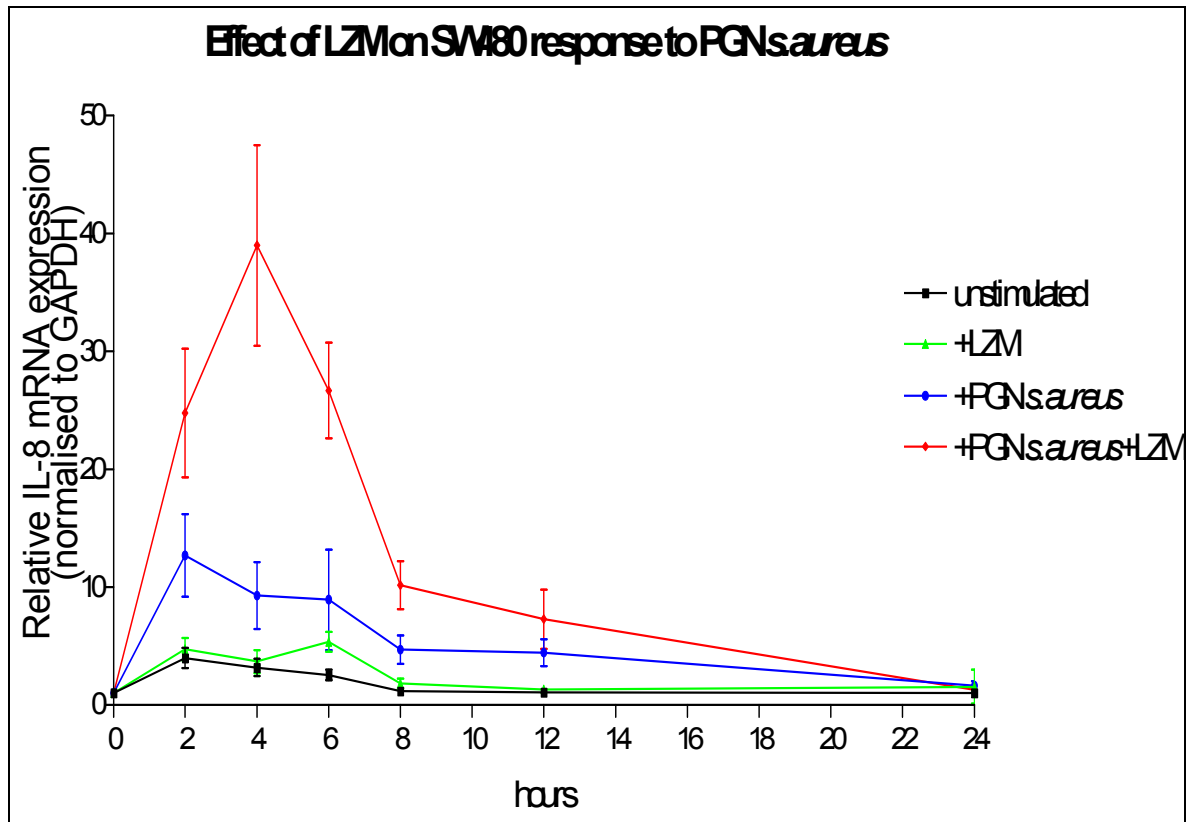


Figure 5.3 Effect of PGN *s.aureus* +/- Lysozyme on IL-8 mRNA expression in SW480 cells – Time course

I also wanted to determine whether LZM acting on PGN from different bacterial species would produce different mucopeptides and thus a different inflammatory response from SW480 cells and so we compared PGN from different species. PGN from *staphylococcus aureus* (a gram positive cocci), and *bacillus subtilis* (a gram positive rod) were initially compared. The same experimental protocol was followed and again the results shown are the results of a minimum of 3 experiments

SW480's were incubated with PGN at a dose of 10µg /ml with or without LZM at a dose of 100mg/ml. The cells were then harvested, RNA extracted and cDNA synthesized. qPCR was performed looking at gene expression. Results were normalized for GAPDH expression and specificity confirmed by ethidium bromide gel visualization.

The results of stimulation with PGN from *S.aureus* showed that LZM alone has no inflammatory response but PGN from *S.aureus* causes a 10 fold induction in IL-8 mRNA expression which is maximal at 4 hours and this effect is multiplied by a factor of four with the addition of LZM (Figure 5.3). This is statistically significant $p<0.05$ at 4 hours ($p=0.027$) but does not reach significance at 2 or 6 hours and by 8 hours the proinflammatory effect was diminished.

We also looked at the expression of TNF α and Human β defensin 2 (HbD2) and the results can be seen in Figures 5.4 & 5.5. LZM alone had no effect on TNF α expression throughout the time period studied. PGN *S.aureus* induced a mean 13 fold induction in TNF α at 4 hours and this was increased to 18 fold with the addition of LZM.

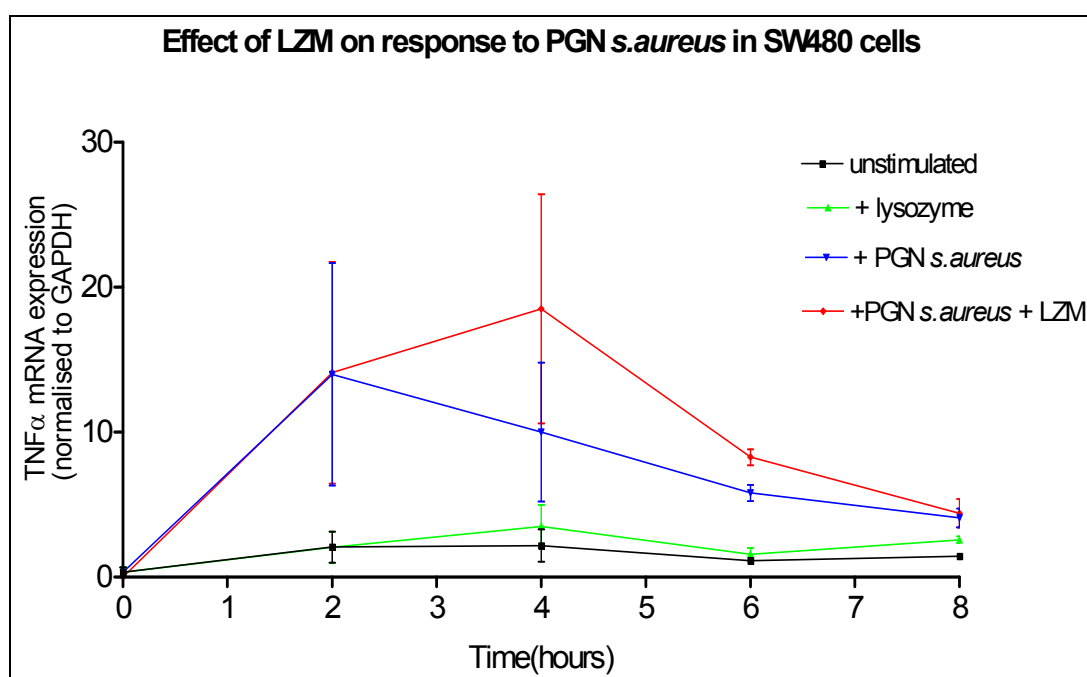


Figure 5.4 - Effect of PGN *s.aureus* +/- Lysozyme on TNF α mRNA expression in SW480 cells – Time course

I also examined the expression of Human β defensin 2 (HbD2) after stimulation with PGN *S.aureus* as I wanted to determine whether LZM acting on PGN induced the transcription of other genes, apart from inflammatory cytokines, that are directly involved in the

generation of antimicrobial molecules such as the defensins. When examining HbD2 expression the increase with LZM was more pronounced at 4 hours from 20 fold to 80 fold induction. At 4 hours this reached statistical significance ($p < 0.05$) but at all other time points it did not. Again LZM alone had no inflammatory activity at any time point.

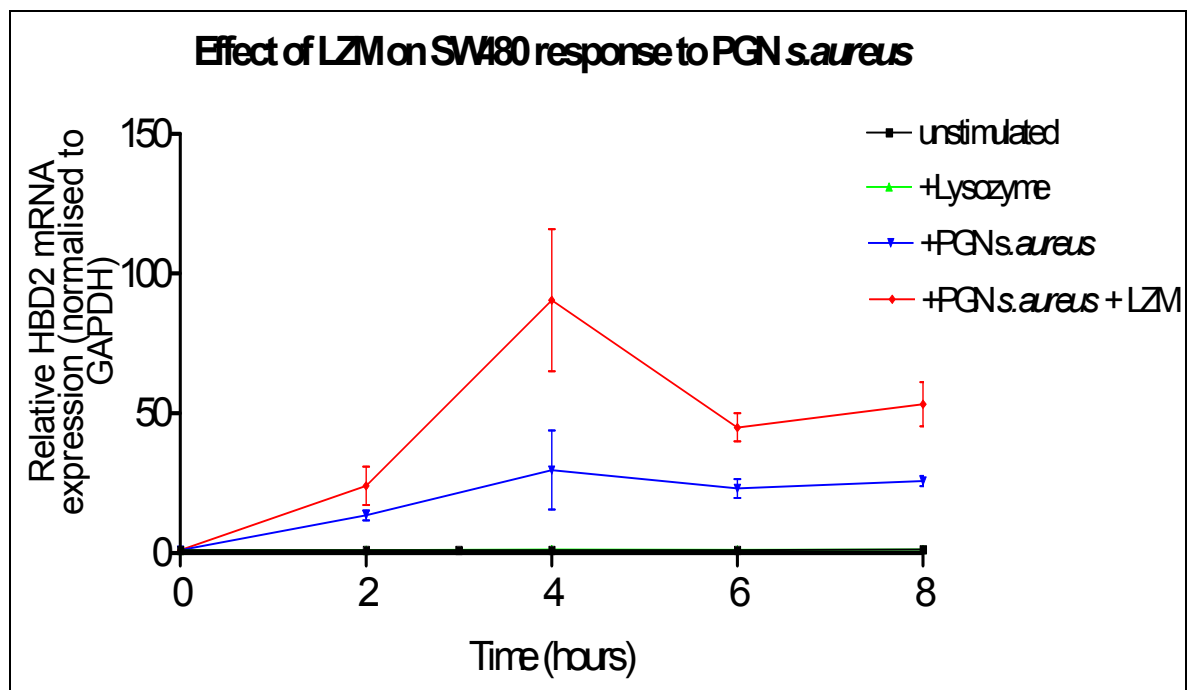


Figure 5.5 - Effect of PGN *s.aureus* +/- Lysozyme on HBD2 mRNA expression in SW480 cells – Time course

5.5 Effect of LZM on SW480 response to PGN *Bacillus Subtilis*

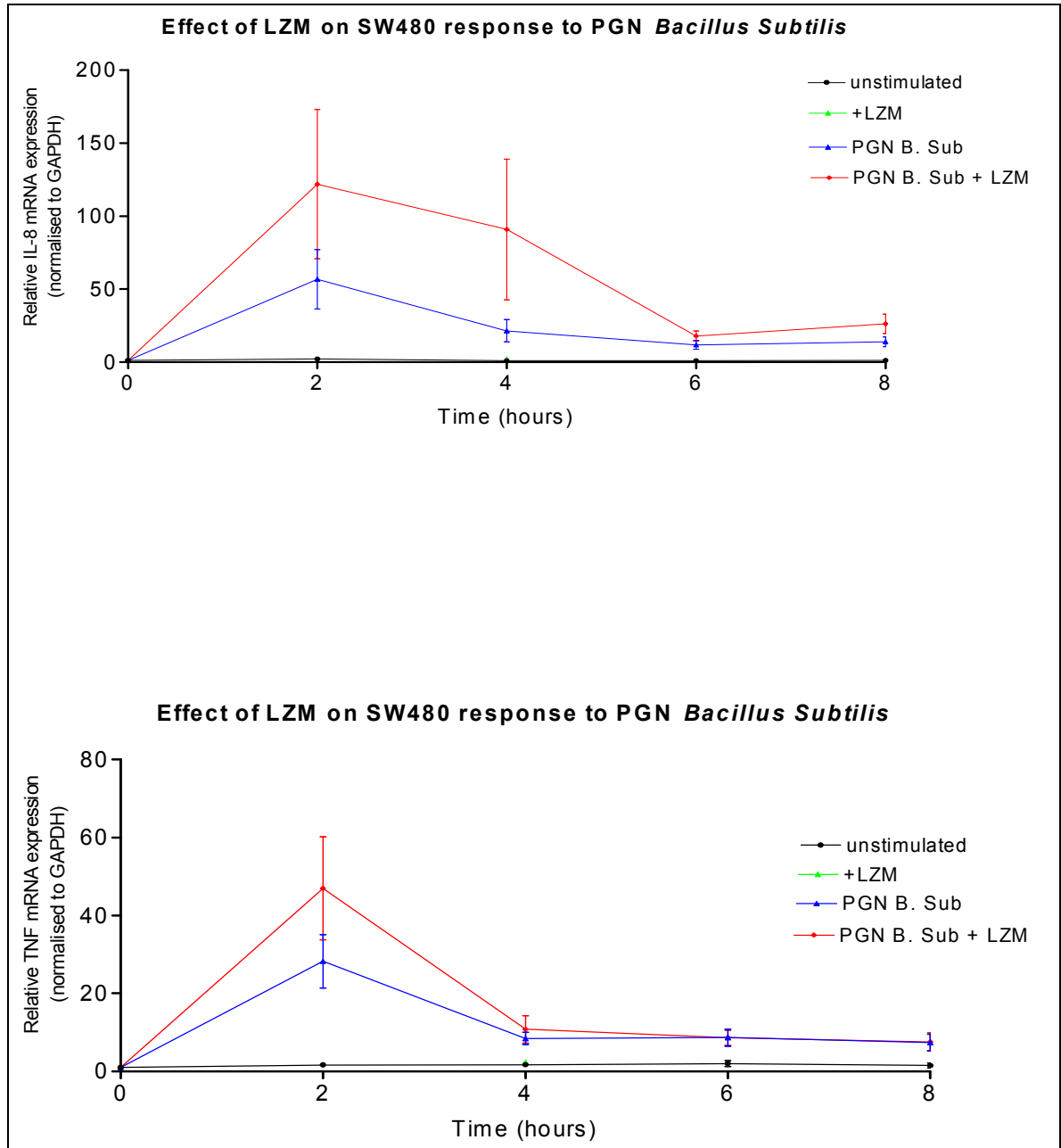


Figure 5.6 - Effect of PGN *B.subtilis* +/- Lysozyme on IL-8 and TNF α mRNA expression in SW480 cells – Time course

When examining PGN from *B.subtilis* a gram negative rod this bacteria was again a powerful stimulant of IL-8 expression and marker of NF- κ B activity in SW480 cells. At 2 hours there was a mean 56 fold induction in IL-8 expression which was augmented to 122

fold induction with the addition of LZM. At 4 hours the effect is still marked with a 21 fold induction being increased to 91 fold with the addition of LZM. The effect of LZM on TNF α is more modest and is only seen at 2 hours. There is a 28 fold induction in TNF α expression which is increased to 50 fold with the addition of LZM. By 4 hours there is no difference between the cells given LZM or not. (Figure 5.6)

In summary when comparing the results of the effect of muropeptides derived from LZM acting on PGN from *S.aureus* and *B.subtilis* in terms of IL-8 and TNF α mRNA I have shown that PGN alone induces an inflammatory response and that this response is enhanced by lysozyme. Furthermore there are differences in different bacterial species derived muropeptides.

5.6 Effect of LZM on SW480 response to *E.coli* LF82

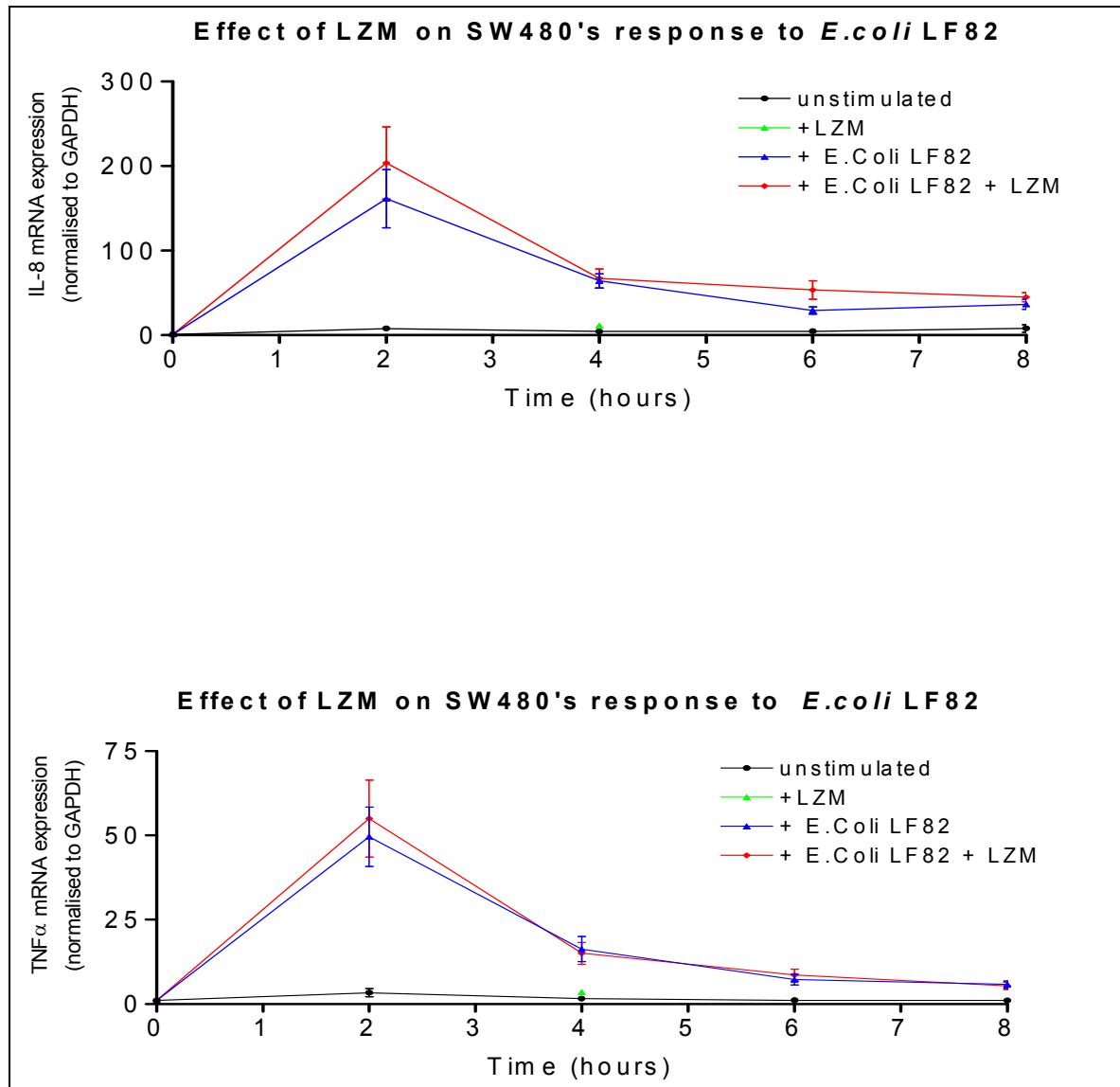


Figure 5.8 - Effect of *E.Coli* LF82+/- Lysozyme on IL-8 and TNF α mRNA expression in SW480 cells – Time course

I also examined the effect of *E.coli* LF82 on SW480 but the results from this are obviously complicated by the fact that it was not pure a pure synthesised PGN and thus the bacteria could trigger the inflammatory pathway by a variety of TLRs including TLR2, TLR 4 and CD14 as well as through PGN breakdown products. I examined *E. Coli* LF82 because as discussed earlier (Ch1.3.4.3) adherent invasive *Escherichia coli* have been proposed as a

causative role in the initiation of Crohn's disease. Strain LF82 was isolated from a chronic lesion of a Crohn's disease patient and has previously been extensively studied (Martin 2004, Darfeuille-Michaud 2004)

Accepting these limitations the results show (Figure 5.8) that *E.coli* LF82 is a powerful inducer of IL-8 causing a mean 161 fold induction at 2 hours. The effect of LZM does increase this response to a mean 204 fold induction at 2 hours whereas again LZM alone has no proinflammatory activity. At 4 hours onwards the response has reduced and there is no difference elicited by the addition of LZM. When examining TNF α mRNA expression this is again maximal at 2 hours but considerably less. At 2 hours there is a mean 50 fold induction in TNF α expression by *E.Coli* alone and the addition of LZM causes this induction to be non significantly higher at 55 fold.

5.7 Effect of LZM on SW480 responses to whole bacteria –other cytokines

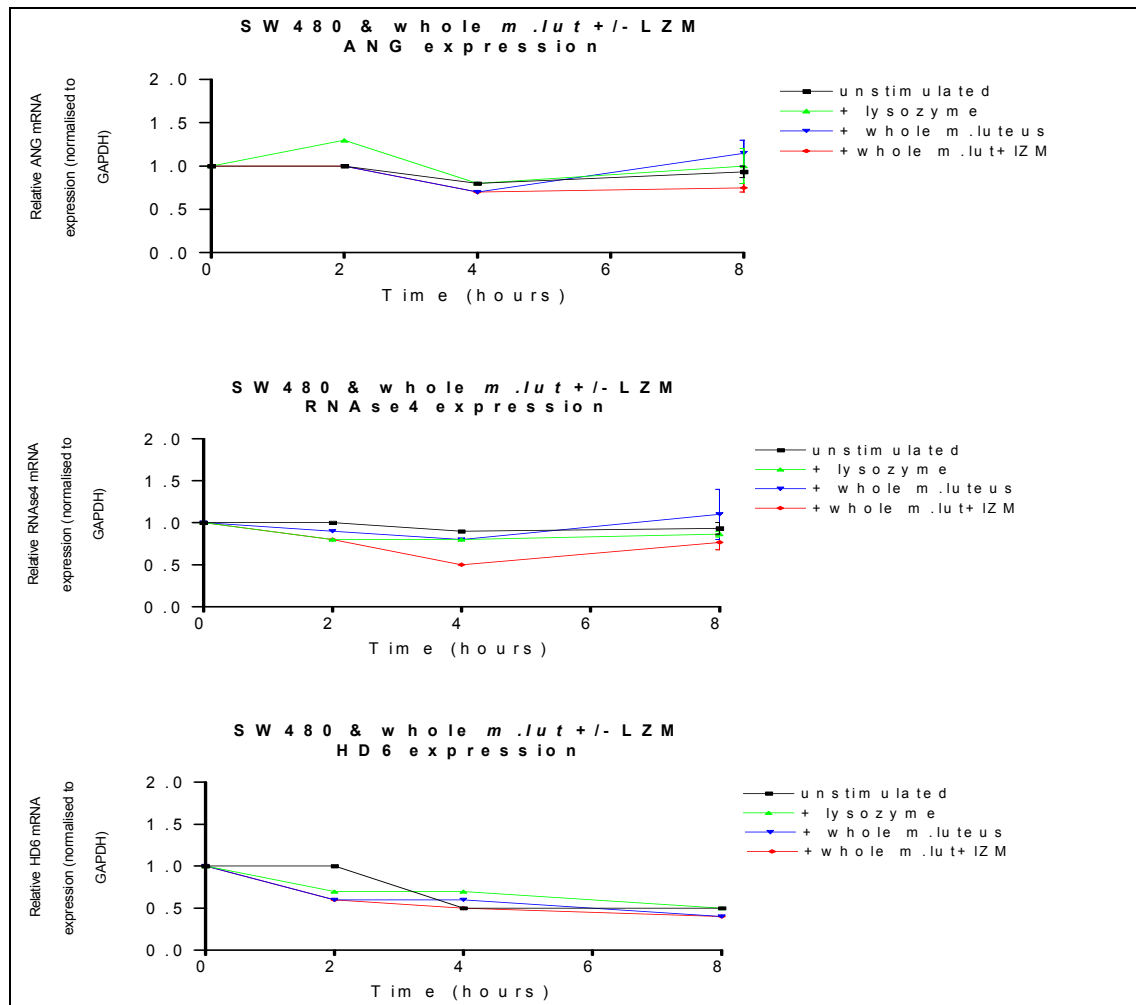


Figure 5.9 - Effect of *micrococcus luteus* +/- Lysozyme on Angiogenin, RNase4 and HD6 mRNA expression in SW480 cells

I studied other cytokines to see if the effect of LZM was a general phenomenon or specific to certain cytokines. Angiogenesis-promoting cytokines have been suggested to play an important role in inflammatory bowel disease (IBD) since they promote inflammation by increasing vascular permeability and mediate tissue repair by activating fibroblasts. (Hooper 2003) Serum angiogenin a protein with both angiogenic and ribonucleolytic activity is elevated in patients with IBD. (Koutroubakis 2004) We looked at Angiogenin, RNase4 and Human Defensin 6 expression to see if these were induced by either whole *m.luteus*, LZM or in combination. Figure 5.9 shows there was no increase in mRNA

expression after stimulation with *m.luteus* and this was unchanged with the addition of LZM over an 8 hour period. I did not examine them further.

5.8 Conclusion

I have shown that LZM, possibly through the generation of bacterial mucopeptides enhances inflammatory signalling in the intestinal epithelial cell line SW480. LZM enhanced whole *M.luteus* induced IL-8 mRNA transcription from SW480 cells and LZM also enhanced PGN *S.aureus* and *B.subtilis* induced IL-8 mRNA transcription. I have also shown that lysozyme alone has no proinflammatory effect on SW480 cells.

My results suggest that cellular inflammatory responses may be dependent on the specific mucopeptides derived from different bacterial species. The different PGN studied, *S.aureus* and *B.subtilis*, produced different responses in terms of magnitude of IL-8 mRNA transcription and differing responses in terms of the other proinflammatory cytokines examined.

LZM acting on PGN *S.aureus* induced significant response in terms of IL-8 and HbD2 mRNA transcription from SW480 cells and the effect of LZM increased this 4 fold whereas the response in terms of TNF α was only doubled with the addition of LZM. This effect was maximal at 4 hours. The response of PGN *B. subtilis* was most marked at 2 hours and the addition of LZM increased the response 2 fold in terms of IL-8 and TNF α but without having any effect on HbD2 expression (figure not shown)

E.Coli LF82 alone induced high levels of TNF α mRNA. Although this was increased by the addition of LZM the effect was less pronounced. This may be relevant as it is possible that this strain is pathogenic in Crohn's disease and LZM may not be so important in terms of the proinflammatory response.

To answer the question does lysozyme allow cells to distinguish between different bacterial species it would be useful to examine all cytokines preferably by micro array and also the response to PGN from a wide variety of Gram negative cocci and rod. I also only measured

mRNA levels not protein levels by ELISA due to cost and time constraints. In future it would be useful to look at both the transcription and translational levels.

PGN alone is known to be sensed through Toll like receptor 2 whereas its breakdown product, after the addition of lysozyme, muropeptide has been demonstrated to be NOD2. It therefore would be crucial and interesting to block Toll like receptor 2 and thereby limit the response of the SW480 to the muropeptide induced response alone. I would expect this to have no effect on the results obtained. We attempted to do this but were unable to entirely block TLR-2. For completeness it would be good to also inhibit TLR 4 and CD14 and show that the response is entirely due to lysozyme mediated breakdown product of PGN and not any other bacterial impurities.

The response of SW480 cells to PGN is augmented by the addition of LZM which presumably breaks down the PGN to produce muropeptides which may be recognized by the NOD2 protein. I could not study the effect of NOD2 in this system but in future it would also be interesting to determine whether the response depended on NOD2. It would be interesting to examine the effect of LZM on bacterial signalling in an intestinal cell line which did not express NOD2 such as HT29 cells and then transfect NOD2 in and then repeat the experiments stimulating them with PGN with or without lysozyme.

Chapter Six

6.0 Discussion

6.1 NOD2 and Crohn's disease

A paradox which has become central to understanding the role of NOD2 in the pathogenesis of Crohn's disease is that in transfection studies, NOD2 mutations, particularly the frameshift mutation are associated with reduced NF- κ B activation in response to MDP stimulation (Inohara 2003, Giardin 2003) whereas Crohn's disease is characterised by increased NF- κ B activation and subsequent downstream effects on cytokine production and immunoregulation (Chamaillard 2003, Podolsky 2002).

Using both experimental animal and human studies several groups, including ourselves have therefore sought to illuminate the mechanism whereby NOD2 mutations predispose to Crohn's disease. Thus far they have not resolved the paradox and the data remains discrepant.

The data I have presented here adds to this.

The results from examining the murine studies reveal the complexity of understanding NOD2 function and its role in the pathogenesis of Crohn's disease. Several of the hypotheses that have been given to explain the observations in the murine studies can be divided conceptually into those suggesting that mutant NOD2 is defective in performing critical functions required for limiting inflammation - "loss-of-function", and those proposing that mutant NOD2 proteins activate pro-inflammatory pathways - "gain-of-function". However, these hypotheses are not mutually exclusive and it may be that to categorise the NOD2 mutation as a loss or gain of function may be an oversimplification of mechanisms in vivo.

6.2 NOD2 Loss of Function or Gain of Function - Murine studies

There have been three reports of NOD2 and TLR function studied in murine models where the NOD2 gene has been modified. Two 'knock-out' murine models have been developed (Pauleu 2003 and Kobayashi 2005) and one murine model with a targeted defect similar to the human Leu1007fsinsC mutation (Maeda 2005) has also been

developed. In all models the mice appear to be healthy and fertile, indicating that the protein has no critical developmental functions.

Watanabe and colleagues (Watanabe 2004) using the NOD2 'knock-out' mouse model, showed that the NOD2 protein acts as an important regulator of NF- κ B activation in response to the TLR2 activation system. They showed that MDP suppressed TLR2 induced NF- κ B activation in wild type cells. However in the NOD2 'knock-out' mouse, this down regulatory process did not occur and proinflammatory cytokines were produced with a T_H1 profile similar to that found in Crohn's disease. This suggests that MDP acting via NOD2 normally inhibits PGN mediated T_H1 responses via TLR2.

Subsequently Maeda and colleagues (Maeda 2005) generated mice with a targeted defect similar to the human Leu1007fsinsC mutation and outwardly they were also healthy and fertile. Firstly, in contrast to transfection studies, they showed that in bone marrow derived macrophages (BMDM) isolated from control and NOD2 mutant mice, MDP induced greater NF- κ B activation and IL-1 β secretion in the mutant mice. They then looked at combined TLR2 and NOD2 activation with PGN and MDP and found no inhibitory function of NOD2 thus not replicating Watanabe's findings. These investigators went on to assess the response to dextran sodium sulphate and showed increased intestinal inflammation, increased IL-1 β secretion and increased mortality rates in the NOD2 mutant mice in comparison to the wild-type mice. These results suggest that the frameshift mutation is a gain-of-function allele and are the opposite of those found in most published human studies.

In contrast however Kobayashi generated NOD2 'knock-out' mice which were also outwardly healthy with no evidence of intestinal inflammation after six months and they stimulated BMDM with TLR2, 3 and 4 ligands (Pam3CS, RNA and LPS respectively) with or without MDP. They showed increased IL-6 and IL-12 secretion in wild type but not mutant mice stimulated with TLR ligands and MDP. They also went on to examine the effect of MDP pre-treatment on the mouse prior to endotoxic shock by LPS injection and showed that pre-treatment with MDP then subsequent LPS was fatal to wild type mice but knock out mice were protected and resistant to LPS challenge. LPS alone showed similar effect and survival in both groups.

They then went on to investigate the role of NOD2 in the innate immune response against bacterial infection. NOD2 $-/-$ mice were more susceptible to oral but not systemic infection with the enteric bacterial pathogen *Listeria monocytogenes*. This was paralleled by decreased expression of the Paneth cell α -defensin, similar to the human findings by Wehkamp (Wehkamp 2004) suggesting that NOD2 plays a pivotal and specific role in protecting against bacterial infections in the intestine.

They conclude by suggesting that mutations in NOD2 may promote Crohn's disease through defective regulation of responses to commensal and or pathogenic bacteria, rather than as an initiating factor for disease.

6.3 NOD2 Loss of Function or Gain of Function - Human studies

The synergistic effects between MDP and TLR's demonstrated by Kobayashi in NOD2 'knock-out' mice have also been demonstrated in human studies. (Yang 2001, Uehara 2004 & van Heel 2005). Yang (2001) showed that MDP alone exhibited a weak ability to induce secretion of inflammatory cytokines but a marked synergistic effect with LPS and LTA was demonstrated in human monocytic THP-1 cells. Uehara (2004) went on to demonstrate that MDP exhibited significant synergistic effects with TLR2, TLR4 and TLR9 agonists to induce the production of proinflammatory cytokines in human monocytic cells. They also showed that NOD2 was responsible for the synergistic effects. Van Heel, as previously discussed in Chapter 4, showed in cryopreserved monocytes that MDP upregulated secretion of TNF α and IL-1 β induced by TLR ligands in wild type cells but that this effect was abolished in NOD2 homozygotes.

The results I show here contribute more data; my results are not definitive but indicate that the mechanisms whereby NOD2 gene mutations contribute to Crohn's disease are likely to be complex. I have shown that cellular activation is reduced in MDP stimulated PBMC expressing mutant NOD2 proteins and these results are consistent with other reports (Ogura 2001, Inohara 2003 & Li 2004). I have, however, also shown that there are conditions whereby mutant NOD2 proteins predispose to the development of enhanced inflammatory responses in PBMC primed by MDP. I have shown that in PBMC not expressing the common NOD2 gene mutations, MDP inhibits the subsequent response to TLR4 activation by LPS; however, in PBMC expressing 2 mutant NOD2 alleles, this effect is reversed. This is the first human data which supports the findings of

Watanabe and colleagues who also demonstrated that mutant NOD2 lacks an inhibitory function. Within the intestinal milieu, it is likely that bacterial antigens continually stimulate immune responses, and I speculate that priming may well be an important phenomenon that determines inflammatory and immune response.

It adds to the theory that intracellular activation of NOD2 by MDP modulates the innate immune response to other pathogens via the extracellular TLR system. Possible explanations for the marked difference between studies (discussed in chapter 4) may be the different cell types used, the variable concentrations of ligands particularly MDP used, or the use of NOD2 expression ‘knock-out’ mice or over expressing transfected cells rather than the more subtle human mutations.

6.4 NOD2 modulates the TLR system

Although traditionally our understanding has been that TLR recognition triggers the innate immune system leading to an inflammatory response, a protective role of TLR activation by intestinal commensal bacteria has been demonstrated. (Rakoff-Nahoum 2004) Under steady state conditions activation of TLRs by commensal microflora was critical for tissue repair and protection against intestinal injury and associated mortality. Furthermore *in vivo* it is likely that innate immune pattern recognition receptors are not triggered by single ligands but rather that complex activation of multiple receptors takes place concurrently due to the many TLR and NOD ligand motifs present on a microbial pathogen. (Sansonetti 2004, Philpott 2004)

I postulate that dual signalling via both TLR and NOD pathways may be necessary for efficient innate immune responses and that in the presence of abnormal NOD2 this process is compromised resulting in an abnormal initial defense against commensal and pathogenic bacteria or an abnormal tolerance mechanism which is critical in maintaining controlled activation of the immune system in the intestine.

6.5 Mechanism of interaction with TLR system

The hypothesis that extracellular TLR and intracellular NOD2 participate together as pattern recognition receptors in regulating the mucosal immune responses to intestinal

microbes is an attractive one; however, the mechanism of interaction between the TLR system and NOD2 activation by MDP is as yet unclear.

It has been demonstrated that NOD2 interacts with RICK to activate NF- κ B activation. Kobayashi showed using mouse cells deficient in RICK reduced cytokine production after stimulation with LPS and PGN but not bacterial CpGDNA indicating that RICK is downstream of TLR2 and 4 but not TLR9 (Kobayashi 2002). Van Heel's human data showing synergistic enhancement of CpGDNA with MDP would also suggest a mechanism other than via RICK. There have been recent papers describing other components of the innate immune pathway which interact with and modify NOD2 function – RIP2 and NEMO (Abbott 2004) NALP3 (Martinon 2004) and GRIM 19 (Barnich 2005) all of which are involved with PAMP recognition and have a downstream effect on NF- κ B activation and which may be implicated in the future.

6.6 The Future

Since 2001 and the discovery of NOD2, a new technique has been developed which has accelerated genetic research. Following the completion of the human genome sequence it has been possible to perform genome wide association studies (GWAS) which provide systematic assessment of the contribution of common variation to disease pathogenesis. These GWAS have resulted in the identification of many novel loci which all confer risk to Crohn's disease. They have also further highlighted the importance of the innate immune system and implicated other new pathogenic pathways.

In 2006 the North American IBD Genetics Consortium provided strong evidence for an association between ileal Crohn's disease and the IL23R gene using genome-wide association studies (Duerr 2006) and this was replicated in a British population in 2007 (Tremelling 2007). IL23 is a critical cytokine in the differentiation of T-helper cells especially their differentiation into Th17 T cells (Steinman 2007). In animal models it has been shown that Th17 Tcell subset mediates chronic inflammatory conditions and furthermore that IL23 is essential for the development of intestinal disease (Hue 2006).

A subsequent GWA reported a SNP in ATG16L1 on chromosome 2, a gene homologous to the essential yeast autophagy gene ATG16, to be associated with Crohn's disease. (Hampe 2007) This was subsequently replicated in the UK. (Prescott

2007) The ATG16L1 protein is expressed in the colon, small intestine and leukocytes and is involved in the autophagy pathway. This is a cellular pathway involved in protein and organelle degradation and mediates resistance to intracellular pathogens. Interestingly autophagy is critical in inhibiting *Mycobacterium tuberculosis* survival in macrophages. (Gutierrez 2004) Increased susceptibility to Crohn's disease secondary to a defective autophagy mechanism (in the case of mutation in ATG16L1) strengthens the hypothesis previously explored that interactions with the intestinal microflora play a significant role in the pathogenesis of Crohn's.

Recently exciting studies by Cadwell and Saitoh have provided further insight into this mechanism by demonstrating the effect the autophagy pathway has on the biology of Paneth cells. (Cadwell 2008, Saitoh 2008) Cadwell and colleagues generated mice with reduced ATG16L1 protein expression and elegantly demonstrated a striking reduction in the secretion of Paneth cell antimicrobial granules into the intestinal lumen. In addition microarray analysis showed that there was up regulation of certain genes associated with the inflammatory response in autophagy deficient Paneth cells. They went on to show in Crohn's disease patients who were homozygous for the ATG16L1 risk allele that they too had similar abnormal Paneth cell granule secretion.

Saitoh demonstrated in mice that ATG16L1 is an essential component of the autophagic machinery responsible for control of the endotoxin-induced inflammatory immune response. (Saitoh 2008) Following stimulation with lipopolysaccharide, ATG16L1-deficient macrophages produced high amounts of the inflammatory cytokines IL-1 β and IL-18. Furthermore mice lacking ATG16L1 were highly susceptible to dextran sulphate sodium-induced acute colitis, which was then alleviated by injection of anti- IL-1 β and IL-18 antibodies thus indicating the importance of ATG16L1 in the suppression of intestinal inflammation.

The last few years have been exciting times in the search for the aetiology of Crohn's disease. The loci identified all encode for genes involved in a variety of mechanisms which give insight into the pathophysiology of Crohn's:– NOD2 which I studied - pathogen recognition, ATG16L1 - autophagy, IL23R - the differentiation of Th17 lymphocytes. The identification of all these loci is however the first step and further studies are needed into their functional implications and also the relative contribution of each pathway to the pathophysiology of Crohn's disease.

Despite these developments the association of the three common NOD2 mutations and Crohn's disease remains the most important genetic susceptibility factor thus far identified. The function of the NOD2 protein as already discussed however is not yet definitively established and the pathogenic mechanisms whereby mutant NOD2 proteins lead to the development of Crohn's disease remains to be defined. The mucosal immune system is the central effector of intestinal inflammation and injury and the evidence indicates that there is dysregulation of mucosal immunity in Crohn's. Under normal situations, the intestinal mucosa is in a state of "controlled" inflammation regulated by a delicate balance of proinflammatory (TNF α , IL1 β , IL-6, IL-12) and anti-inflammatory cytokines (IL-4, IL-10, IL-1). As the mucosal immune system is continually responding to commensal bacteria and rarely activates the systemic immune system, experiments which have thus far been done in both transfected cell lines and human PBMC may be of limited relevance to how NOD2 causes intestinal inflammation.

Until very recently, the function of Paneth cells, first described over 100 years ago, had remained obscure. Recently, however, the importance of these cells has been highlighted firstly with the identification of NOD2 in them and more recently with ATG16L1. Further studies are needed therefore into the relative contribution of the effect of mutations in both NOD2 and ATG16L1 on Paneth cell function and I think will be critical to our understanding of the pathophysiology of Crohn's.

The other critical unanswered question is how the environment, for example smoking interacts with the genetic make up to determine Crohn's susceptibility and this will be imperative to clarify.

Finally it is worth remembering that despite the great advances in the understanding of the pathophysiology of Crohn's there has been limited impact on either the diagnosis, management or treatment of Crohn's disease. Newer therapeutic targets have been identified (such as IL23) but the transition from the lab bench to the clinical setting has yet to be made. The most likely first impact on patient's lives will be of tailoring treatment strategies to the individual's genetic make up.

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Appendix A: The allele frequencies of Crohn's disease-associated NOD2 variants

Study, First author (year)	Allele frequencies (%)						Patients with at least one NOD2 risk allele					
	Arg702Trp		Gly908Arg		Leu1007fsinsC		Crohn's disease	Healthy controls	Ulcerative colitis			
<i>Caucasians, non-Jewish descent</i>												
Ogura (2001)	-	-	-	-	-	-	8.2	4	3	-	-	
Abreu (2002) 2 cohorts	8.1	-	2.9	5.6	-	0.9	5.8	-	1.7	34.4	-	10.9
Ahmad (2002)	12.5	5.2	-	3.3	1.4	-	9.4	1.6	-	38.5	15.8	
Cuthbert (2002)	9.1	3.5	3.7	3.3	0.6	1.6	5.4	2.1	1.5			14.6
Hampe (2002)	4.5	2.7	-	0.9	1.2	-	2.7	1.2	-			
Lesage (2002)	10.8	4.4	3.1	6.1	1	0.3	10.6	1.9	1.3	41.5	14.6	8.8
Mascheretti (2002)	7.9	4.3	-	3.8	2	-	7.6	3.5	-	31.3		
Radlmayr (2002)							13.9	1.7	2.1			

Study, First author (year)	Allele frequencies (%)						Patients with at least one NOD2 risk allele (%)					
	Arg702Trp		Gly908Arg		Leu1007fsinsC		Crohn's disease	Healthy controls	Ulcerative colitis			
<i>Caucasians, non-Jewish descent, continued</i>												
Vermeire (2002) B+C	11.5	3.2	-	5	0.6	-	7.8	0.9	-	38.7	8.7	
Brant (2003)										3.9		
Cavanaugh (2003)	11.2	4.5	0	2.4	0.7	0	6.7	1	0	36.7	12.5	0
Croucher (2003)	8.5	6.1	-	4.1	1.3	-	11	4.2	3.1	39.7	19.3	
Helio (2003)	3.3	1.8	1.5	0.6	0	0	5	1.7	3	15.5	6.7	9.1
Louis (2003)										45.5		
Mirza (2003)										34.4	14.3	
Murillo (2003)							8.5	1				
Palmieri (2003)	10.6	6.0	3.1	8.5	2.3	3.1	9.6	1.4	3.1			
Roussomoustakaki (2003)							2.7	1.5	0			
Sugimura (2003)	8.4	3.5	-	8.4	3.5	-	5.1	1.4		30.1	13.3	

Study, First author (year)	Allele frequencies (%)						Patients with at least one NOD2 risk allele (%)		
	Arg702Trp		Gly908Arg		Leu1007fsinsC				
	Crohn's disease	Healthy controls	Ulcerative colitis	Crohn's disease	Healthy controls	Ulcerative colitis	Crohn's disease	Healthy controls	Ulcerative colitis
<i>Caucasians, non-Jewish descent, continued</i>									
Tomer (2003)	2.9	0.7	-	5.7	2.9	-	8.6	1.8	11.0
van der Linde (2003)							13.9	1.9	1.6
Mendoza (2003)	13.7	4.3	-	8.3	2.1	-	14.2	4.3	10.7
Annese (2004)	8.8	3.9	-	6.9	2.0	-	8.3	0.7	13.4
Nunez (2004)	6.7	5.8	-	4.5	1.0	-	4.5	1.0	15.2
Buning (2004)	7.2	3.6	2.1	4.2	2.1	2.1	12.2	2.1	15.5
Giachino (2004)	8.9	5.6	10.9	4.9	1.4	2.7	6.3	2.2	18.6
Newman (2004)	10.3	5.0	-	3.7	2.0	-	4.9	3.0	33.3

Study, First author (year)	Allele frequencies (%)						Patients with at least one NOD2 risk allele (%)		
	Arg702Trp		Gly908Arg		Leu1007fsinsC				
	Crohn's disease	Healthy controls	Ulcerative colitis	Crohn's disease	Healthy controls	Ulcerative colitis	Crohn's disease	Healthy controls	Ulcerative colitis
<i>Caucasians, Jewish descent</i>									
Zhou (2002)	5.1	4.5	-	8.3	5.5	-	6.1	3.6	-
Fidder (2003)	5.3	-	2.9	7.9	-	2.9	2.4	-	0.7
Sugimura (2003)	4.5	3.8		7.6	5.1		5.8	1.3	
Newman (2004)	4.9	3.2		7.3	4.3		8.4	3.7	
Tukel (2004)	6.9	2.8		8.3	4.3		5.6	3.0	
Karban (2004)									
							38.0		9.1

Study, First author (year)	Allele frequencies (%)									Patients with at least one NOD2 risk allele (%)		
	Arg702Trp			Gly908Arg			Leu1007fsinsC					
<i>Asians</i>	Crohn's disease	Healthy controls	Ulcerative colitis	Crohn's disease	Healthy controls	Ulcerative colitis	Crohn's disease	Healthy controls	Ulcerative colitis	Crohn's disease	Healthy controls	Ulcerative colitis
	Inoue (2002)	0	0	0	0	0	0	0	0	0	0	0
	Croucher (2003)	0	0		0	0		0	0		0	
	Leong (2003)	0	0	0	0	0	0	0	0	0	0	0
	Sugimura (2003)	0	0		0	0		0	0		0	
	Guo (2004)				3.3	0.3	1.3	7.1	0.6	2.7		

Appendix B: The Vienna Classification of Crohn's disease

Age at diagnosis ¹ :	A1, <40 years A2, ≥40 years
Location ² :	L1, Terminal ileum ³ L2, Colon ⁴ L3, Ileo-colon ⁵ L4, Upper gastrointestinal ⁶
Behavior:	B1, Non-stricturing non-penetrating (i.e. inflammatory) ⁷ B2, Stricturing ⁸ B3, Penetrating ⁹

¹. The age when diagnosis of Crohn's disease was first definitively established by radiology, endoscopy, pathology or surgery.

². The maximum extent of disease involvement at any time before the first resection. Minimum involvement for a location is defined as any aphthous lesion or ulceration. Mucosal erythema and oedema are insufficient. For classification, at least both a small bowel and large bowel examination are required.

³. Disease limited to the terminal ileum (the lower third of the small bowel) with or without spill over into caecum.

⁴. Any colonic location between caecum and rectum with no small bowel or upper gastrointestinal (GI) involvement.

⁵. Disease of the terminal ileum with or without spill over into caecum and any location between ascending colon and rectum.

⁶. Any disease location proximal to the terminal ileum (excluding the mouth) regardless of additional involvement of the terminal ileum or colon.

⁷. Inflammatory disease which never has been complicated at any time in the course of disease. Also known as 'uncomplicated' or 'inflammatory'

⁸. Stricturing disease is defined as the occurrence of constant luminal narrowing demonstrated by radiologic, endoscopic or surgical-pathologic methods with prestenotic dilatation or obstructive signs/ symptoms without presence of penetrating disease at any time in the course of disease.

⁹ Penetrating disease is defined as the occurrence of intra-abdominal or perianal fistulas, inflammatory masses and/or abscesses at any time in the course of disease. Perianal ulcers are also included. Excluded are postoperative intra-abdominal complications and perianal skin tags.

Appendix C: List of reagents and suppliers

	Name of reagent	Supplier
1	100x Antibiotic antimycotic solution: 10 000 U penicillin, 10 mg streptomycin, and 25 µg amphotericin B per mL	Sigma
2.	10x Trypsin-EDTA solution: 5.0 g porcine trypsin, 2.0 g EDTA·4 Na in 0.9% NaCl	Sigma
3.	5X First Strand Buffer [250 mM Tris-HCl (pH 8.30, 375 mM KCl, 15 mM MgCl ₂]	Invitrogen
4	Agarose	Sigma
5	Chloroform	Sigma
6	Deoxyadenosine triphosphate (dATP) 100 mM	Invitrogen
7	Deoxycytidine triphosphate (dCTP) 100 mM	Invitrogen
8.	Deoxyguanosine triphosphate (dGTP) 100 mM	Invitrogen
.	Deoxythymidine triphosphate (dTTP) 100 mM	Invitrogen
9.	Diethyl-pyrocabonate (DEPC)	Sigma
10.	DMEM/F12 medium	Sigma
13.	Dulbecco's modified Eagle medium (DMEM)	Sigma
14.	EDTA (pH 8.0) Ethylenediaminetetraacetic acid	Sigma
15.	Ethanol	VWR
16.	Ethidium bromide	Sigma
17.	Ficoll 400	Sigma
18.	Hanks' balanced salt solutions (HBSS)	Sigma
19.	Histopaque® 1077	Sigma
19.	Isopropanol	VWR
20.	L-Glutamine, 200 mM	Sigma
70.	Magnesium chloride (MgCl ₂)	Sigma
21.	Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (200 units/ µL)	Invitrogen
22.	Nuclease-free water	Invitrogen
23.	Oligo(dT) ₁₂₋₁₈ primer	Invitrogen
24.	Phosphate Buffered Solution (PBS)	Sigma
90.	Q Solution	Qiagen

Sense and antisense primers	Thermo Electron GmbH
SYBR green	Biogene
25. Trizol reagent	Invitrogen

List of Suppliers:

Amersham Biosciences UK Limited

Amersham Place
Little Chalfont
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